LÍVIA MARIA SILVA ATAÍDE

Tetranychus evansi evades plant defence

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Entomologia, para obtenção do título de *Doctor Scientiae*.

VIÇOSA
MINAS GERAIS – BRASIL
2013

Ficha catalográfica preparada pela Seção de Catalogação e Classificação da Biblioteca Central da UFV

T

A862t
2013

Ataíde, Lívia Maria Silva, 1983-
Tetranychus evansi evades plant defence / Lívia Maria Silva Ataíde. – Viçosa, MG, 2013.
x, 116 f. : il. ; 29 cm.

Orientador: Ângelo Pallini Filho.
Tese (doutorado) - Universidade Federal de Viçosa.
Inclui bibliografia.

1. Tomate - Resistência a doenças e pragas. 2. Relação inseto-planta. 3. *Tetranychus urticae*. 4. *Tetranychus evansi*. 5. *Phytoseiulus longipes*. I. Universidade Federal de Viçosa. Departamento de Entomologia. Programa de Pós-Graduação em Entomologia. II. Título..

CDD 22. ed. 635.642

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APROVADA: 21 de junho de 2013.

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Aos meus pais:

Geraldo Honório Oliveira Mascarenhas

&

Mônica Cristina Silva Mascarenhas

AGRADECIMENTOS

Ao prof. Ângelo Pallini pela oportunidade de desenvolver meu projeto de doutorado no laboratório de Acarologia e pela atenção, paciência e orientação durante esses quatro anos.

Ao co-orientador Dr. Arne Janssen por ter me guiado durante a realização deste projeto e principalmente por sempre me estender a mão, seja no Brasil ou na Holanda, durante todo esse tempo, contribuindo grandemente para minha formação profissional e amadurecimento pessoal.

Aos co-orientador prof. Eraldo Lima, a co-orientadora Dr. Madelaine Venzon e prof. Maria Goreti de Oliveira pelas valiosas críticas que contribuíram para o meu crescimento profissional. Um agradecimento especial ao prof. Eraldo por ter sido o primeiro a me abrir as portas desta Universidade.

Aos colegas do laboratório de Acarologia: Ana, Cleide, Marcus, Caelum, Cleber, Felipe, João e Jussara.

Aos demais colegas do departamento de Entomologia e principalmente aos amigos Daniel, Carla, Paulo, Juliana e Damares.

Ao Institute for Biodiversity and Ecosystem Dynamics (IBED) da University of Amsterdam (UvA) por permitir a realização de parte dos meus experimentos durante o meu período de doutorado sanduíche nesta instituição.

A Universidade Federal de Viçosa (UFV) e ao Programa de Pós-graduação em Entomologia pela oportunidade de realização do curso.

A FAPEMIG e CAPES pela concessão da bolsa de estudos.

Aos meus queridos pais, irmãos, tios e minha querida avó pela presença em todos os momentos.

A todos, muito obrigada!

BIOGRAFIA

Lívia Maria Silva Ataíde, filha de Mônica Cristina Silva Mascarenhas e Geraldo Honório Oliveira Mascarenhas, nasceu em Belo Horizonte, Minas Gerais, em 18 de setembro de 1983. Em dezembro de 2005, graduou-se em Biologia, pelo Centro universitário Metodista Izabela Hendrix, Belo Horizonte-MG. Durante o período de graduação, de 2002 a 2005 foi estagiária da FUNASA (Fundação Nacional de Saúde), UFMG (Universidade Federal de Minas Gerais), FIOCRUZ (René Rachou) e CTRS (Central de Tratamento de Resíduos Sólidos). Após esse período lecionou por quase dois anos, disciplinas de Ciências e Biologia em escolas públicas de Belo Horizonte. Em agosto de 2007 ingressou na Universidade Federal de Viçosa (UFV), Viçosa-MG para o curso de Mestrado em Entomologia que foi finalizado em julho de 2009. Em agosto de 2009, iniciou o curso de Doutorado em Entomologia nesta mesma Universidade.

SUMÁRIO

RESUMO

ATAÍDE, Lívia Maria Silva, D.Sc., Universidade Federal de Viçosa, junho de 2013. ***Tetranychus evansi* se evade da defesa da planta.** Orientador: Ângelo Pallini Filho. Co-orientadores: Arnoldus Rudolf Maria Janssen, Madelaine Venzon, Eraldo Rodrigues de Lima e Derly José Henriques da Silva.

Ácaros fitófagos podem induzir ou suprimir as defesas da planta. A maioria das linhagens da espécie *Tetranychus urticae* induz as defesas reguladas pela rota do ácido jasmônico (JA) e ácido salicílico (SA) e essa indução tem sido correlacionada com a diminuição do desempenho reprodutivo de ácaros em plantas de tomate. Diferentemente, o ácaro vermelho *Tetranychus evansi* suprime as defesas reguladas pelas rotas do JA e SA e essa supressão se correlaciona com o aumento do desempenho dos ácaros de ambas as espécies. Isso significa que o desempenho reprodutivo de *T. urticae* e *T. evansi* é maior em folhas de tomate que foram previamente atacadas pelo ácaro supressor. Além disso, *T. evansi* produz uma densa teia sobre suas colônias, provavelmente para evitar competidores. Considerando que *T. evansi* suprime as defesas da planta, essa supressão pode ser vantajosa não apenas para os indivíduos de uma mesma espécie, mas também para outros herbívoros, principalmente quando essa supressão ocorre sistemicamente e em partes da planta não cobertas pela teia. Do mesmo modo, a indução de defesas pelo *T. urticae* pode afetar negativamente outros herbívoros. Portanto, nesta tese foi investigado se *T. evansi* suprime e *T. urticae* induz as defesas diretas de plantas de tomate apenas no local de ataque ou sistemicamente em partes não atacadas. Como resultado, foi encontrado que *T. evansi* manipula as defesas principalmente no seu local de ataque, porque o seu desempenho foi afetado apenas nos folíolos atacados e não foi afetado nos folíolos adjacentes. Portanto, sugere-se que estas duas estratégias (a produção da teia e a supressão das defesas de plantas apenas no seu local de ataque) podem evitar que outros herbívoros se beneficiem da supressão de defesa da planta promovida pelo *T. evansi*. Além disso, nesta tese foi também demonstrado que *T. evansi* manipula a indução de defesas diretas de outra planta, o feijão (*Phaseolus vulgaris*). Essa manipulação de defesas por *T. evansi* em plantas de feijão parece beneficiar *T.* urticae, pois o mesmo teve seu desempenho

reprodutivo aumentado em folhas infestadas juntamente com o *T. evansi*. Devido às semelhanças desses resultados com resultados anteriores de supressão utilizando plantas de tomate, possivelmente, a capacidade de *T. evansi* de suprimir as defesas da planta é uma estratégia utilizada por esse herbívoro em várias plantas e não apenas no tomate e no feijão. Finalmente, tem sido sugerido que a supressão de defesas poderia beneficiar a planta e não o herbívoro. Isso, porque os inimigos naturais são na maioria das vezes sensíveis aos compostos produzidos pela planta como defesas diretas, e, portanto, a supressão dessas defesas pode beneficiar os inimigos naturais. Ou seja, é possível que a supressão das defesas por *T. evansi* e a indução por *T. urticae* afete os próprios herbívoros e seus inimigos naturais. Portanto, foi também investigado como a indução de defesas reguladas pela rota do JA ocasionada pelo ácaro *T. urticae* e a supressão pelo *T. evansi* podem afetar seu desempenho reprodutivo e o desempenho de seu ácaro predador, o *Phytoseiulus longipes*. Os resultados demonstram que o desempenho reprodutivo dessas duas espécies de ácaros e do ácaro predador foi afetado negativamente pelas defesas reguladas pela rota do JA. Além disso, os predadores preferem se alimentar de ovos provenientes de presas que se alimentaram em plantas sem o JA do que ovos provenientes de plantas com JA. Isso sugere que a supressão de defesas por *T. evansi* pode tornar seus ovos mais vulneráveis à predação. Portanto, ainda é uma questão em aberto, se a supressão das defesas de plantas por este ácaro é uma boa estratégia ou não. O objetivo desta tese foi obter maior clareza quanto aos custos e benefícios da supressão de defesa de plantas pelo *T. evansi* em comunidades seminaturais, conectando ecologia e biologia molecular. Reunir essas duas áreas de pesquisa tradicionalmente separadas dentro da comunidade científica visa fornecer novas perspectivas sobre como produzir e utilizar tomates geneticamente modificados e como manipular inimigos naturais, favorecendo assim o controle de pragas na agricultura.

ABSTRACT

ATAÍDE, Lívia Maria Silva, D.Sc., Universidade Federal de Viçosa, June, 2013. ***Tetranychus evansi* evades plant defence.** Advisor: Ângelo Pallini Filho. Co-advisors: Arnoldus Rudolf Maria Janssen, Madelaine Venzon, Eraldo Rodrigues de Lima and Derly José Henriques da Silva.

Spider mites are known to induce or suppress plant defences. For instance, most strains of *Tetranychus urticae* induce plant defences regulated by jasmonic acid (JA) and salicylic acid (SA) pathways and this response has been correlated with a reduction in their reproductive performance on tomato plants. In contrast, the red spider mite *Tetranychus evansi* suppresses the JA and SA defences and both spider mite species were found to perform much better on tomato leaves that were previously attacked by the suppressor mite. In addition, *T. evansi* spins a dense web over its colonies, which has been suggested as a strategy to hinder competitors. Given that *T. evansi* suppresses plant defences and that *T. urticae* induces it, the plant defences suppressed by the former, may be advantageous not only to conspecific mites, but also to other herbivores; especially if the plant response is not local, but systemic throughout the plant and if the plant parts attacked by *T. evansi* are not covered by the web. On the contrary, plant defences induced by the *T. urticae* may negatively affect other herbivores. Hence, I have investigated whether *T. evansi* and *T. urticae* affect induced tomato plant defences locally or systemically. The results show that *T. evansi* seems to manipulate plant defences mainly on its feeding site, because its oviposition performance was only positively affected at the site of its attack, but not in adjacent tomato leaflets. It suggests that these two strategies together (the spin of its web and the suppression of plant defences by *T. evansi* only at its feeding site) can confer advantages to avoid other herbivores of profiting from the suppression of plant defence. In addition, I also show that *T. evansi* manipulates induced plant defences in another plant, the common bean (*Phaseolus vulgaris*). Since *T. urticae* had a higher reproductive performance when sharing a leaf with *T. evansi*, the manipulation of plant defences by *T. evansi* on beans seems to benefit *T. urticae*. Due to the similarities of my

results with previous reports of suppression using tomato plants, the *T. evansi* ability to suppress plant defences might be a widespread mechanism and employed on other plants and not only in tomato and beans. Finally, it has been suggested that suppression could even benefit the plant rather than the herbivore. Because natural enemies can be sensitive to compounds produced by the plant as direct plant defences, the natural enemies may benefit from suppression of these defences. Hence, suppression of plant defences by *T. evansi* and the induction by *T. urticae* may affect not only themselves, but also their natural enemies. Therefore, I also investigated how the induction of JA defences by the spider mite *T. urticae* and suppression by *T. evansi* can affect their reproductive performance and the performance of their predatory mite, *Phytoseiulus longipes*. My data showed that the reproductive performance of both spider mite species and the predatory mite was negatively affected by JA defences. In addition, we also show that predatory mites eat more prey eggs when these came from JA-undefended plants than from JA-defended plants. It suggests that defence suppression by *T. evansi* can backfire, since it makes it eggs more vulnerable to predation. Therefore, it is still an open question, whether the suppression of plant defences by this spider mite is the best strategy or not. In this thesis, I aimed to get a better insight in the costs and benefits of plant defence suppression by *T. evansi* within simple semi-natural communities linking ecology and molecular biology. These two fields of research traditionally work separately in the science. To bring them together can provide novel insights into how to use and breed tomato plants and how to manipulate the natural enemies to favour pest control in agriculture.

General Introduction

It is known that plants defend themselves against herbivore arthropods, using a combination of physical and chemical traits that reduce the performance of such herbivores (Schoonhoven *et al.*, 2005). Furthermore, some plants constitutively accumulate high levels of defence compounds, while others can be induced to produce plant defensive compounds that turn them more resistant to subsequent attack by a broad range of herbivores (Karban & Baldwin, 1997; Arimura *et al.*, 2005). These so-called induced defences are characterized by the accumulation of toxins and digestive inhibitors, collectively discouraging the herbivore to continue feeding. Nevertheless, a plant cannot only directly defend itself against herbivores, but also indirectly by attracting foraging natural enemies via distinct odor signals (Karban & Baldwin, 1997).

Due to its relevance for agriculture, the study of how plants defend themselves against herbivores has been the focus of studies for several decades. However, it is also known that while plants have evolved sophisticated mechanisms to overcome herbivore attack, herbivores in turn have evolved counter-adaptations to overcome them and to increase their own performance (Karban & Agrawal, 2002). Hence, if for several decades the focus was to study how plants defend themselves against herbivores, recently there is a shift of focus to the response of arthropod herbivores to plant defences.

Herbivores can sequester the defensive chemicals of the host plant for their own protection (Karban & Baldwin, 1997) or dispose of them via (enzymatic) degradation, modification or secretion (Schoonhoven *et al.*, 2005). Moreover, some herbivores and pathogens have adapted to manipulate plants to their own benefit, such that establishment of induced defences is reduced or inhibited. For instance, Sarmento *et al.* (2011a) found that the red spider mite *Tetranychus evansi* is able to manipulate tomato plant defences, reducing or inhibiting the production of defensive compounds.

Considering that plants and herbivores experience selection pressure (Sabelis *et al.*, 1999) and the effects of plant defence and herbivore attack must consider all defence and attack strategies simultaneously. Furthermore, *T. evansi* may have invested heavily in traits necessary to manipulate plant defences and such manipulation is expected to promote ecological costs. For instance, by decreasing plant defence, herbivores may facilitate competitors which may profit from these investments without having invested in such traits. Indeed, the closely related spider mite *Tetranychus urticae* also profits from the increase in plant quality caused by the attack of *T. evansi* (Sarmento *et al.*, 2011b). At the same time, *T. urticae* does induce direct and indirect plant defences in tomato (Ament *et al.*, 2004; Kant *et al.*, 2004) and the performance of *T. evansi* is reduced on plants induced by *T. urticae* (Sarmento *et al.*, 2011a). If suppression of plant defences by *T. evansi* makes plants more profitable to other herbivores, it suggests that suppression of plant defences can backfire.

Other ecological costs might be involved in the suppression of plant defence by *T. evansi*, which may, for instance, affect the third trophic level through indirect plant defences. If it is known that induction of plant defence can negatively affect natural enemies (Thaler, 1999), in the opposite suppression can have positive effects on the natural enemies of herbivores. Indeed, it has been suggested that suppression could even benefit the plant rather than the herbivore. Kahl *et al.* (2000) reported that *Manduca sexta* suppressed the production of nicotine in tobacco plants, but increased the production of volatiles involved in the attraction of parasitoids of this herbivore. Since nicotine is toxic to the parasitoid, the authors suggested that the inhibition of nicotine production may represent an adaptive response of the plant against a herbivore. We therefore argue here that suppression of plant defences by *T. evansi* and the induction by *T. urticae* may affect not only the spider mites themselves, but also their natural enemies. Therefore, although suppression of plant defence by *T. evansi* correlates with an increased oviposition, it is still an open question whether the suppression of plant defences by this spider mite is the best strategy or not.

The experimental system

The red spider mite *T. evansi* is a specialist on Solanaceae (Migeon *et al.*, 2009) and in the late 1970s and the early 1980s, when the cultivation of tomato but also tobacco, potato and eggplant was increasing rapidly in Brazil, it was considered a tomato pest, especially in north-eastern. However, for unknown reasons, *T. evansi* is nowadays not a priority pest on tomato in this region anymore. It has been suggested that it originated in South America (Migeon *et al.*, 2009) and genetic studies are consistent with this hypothesis (Boubou *et al.*, 2011; Boubou *et al.*, 2012). Infested commercial crops are overexploited very rapidly and it has become one of the most severe pests of Solanaceae in Africa (Saunyama & Knapp, 2003) and more recently in Europe. It has reached Spain in 1995, Portugal in 2000, France in 2005, Italy in 2006 and Greece in 2007 (De Moraes & McMurtry, 1987; Ferragut & Escudero, 1999; EPPO, 2004; Tsagkarakou *et al.*, 2007). The biological control of spider mites with predators has shown to be a good alternative to pesticides. However, because until now *T. evansi* cannot be controlled by commercial available predators it has become a serious threat to tomato crops.

The two-spotted mite *T. urticae* Koch (Acari: Tetranychidae) is a closely related spider mite, but it is an extreme generalist and an economically important pest of a wide range of plants, including fruits, vegetables, grains and ornamental crops (Li *et al.*, 2002). According to Bolland *et al.* (1998) *T. urticae* has been recorded from over 900 plant species from 124 plant families. Recently, it has been suggested to be found on over 1,100 plant species belonging to more than 140 different plant families (Grbic *et al.*, 2011). *T. urticae* is known for its ability to rapidly adapt to novel hosts (Magalhaes *et al.*, 2007), which makes this mite a serious pest across the globe.

Spider mites form colonies on its host plant, which it covers with web. *T. evansi* produces higher amount of web when compared with other *Tetranychus* species, such as *T. urticae* (Ferragut & Escudero, 1999). Since it has been suggested that the web can protect the eggs from predation (Sabelis & Bakker, 1992), perhaps it has been difficult to control *T. evansi* using commercial predators, because the predation efficiency of many predators are negatively affected by the presence of the web (Venzon *et al.*, 2009). *Phytoseiulus longipes* is a predatory mite that has recently been suggested as a promising control agent of *T. evansi* because it can cope with the web produced by *T. evansi* (Furtado *et al.*, 2007; Silva *et al.*, 2010). Other phytoseiids, such as *Phytoseiulus persimilis* Athias-Henriot (Acari: Phytoseiidae) and *Neoseiulus californicus* McGregor (Acari: Phytoseiidae), which are often used as control agents for *T. urticae*, cannot control *T. evansi* effectively (de Moraes & McMurtry, 1986). Furthermore, since the web is made with protein, the silk of the web must be costly and therefore, it is expected that mites will fine-tune the production of web depending on the environment. Sarmento *et al.* (2011b) have shown that the web can also serve to hinder competitors and that the production of web is increased in the presence of damage or other cues associated with the mite *T. urticae*. All these features makes *T. evansi* an herbivore with unique properties that deserves considerable attention to promote its control as a pest all over the world.

Thesis overview

In this thesis, I have focused on the induction of direct plant defences by the spider mite *T. urticae* and on the suppression by *T. evansi* and how these defences can affect the reproductive performance of both spider mite species and their predatory mite. In doing so, I aimed to get a better insight in the costs and benefits of defence suppression within simple semi-natural communities. In the following section, I will briefly summarize the questions and results addressed in each chapter of this thesis.

CHAPTER 1: Local and systemic effects of suppression of plant defences by *Tetranychus evansi* and induction by *Tetranychus urticae*

Given that *T. evansi* suppresses plant defences and that *T. urticae* induces it, the plant defences suppressed by the former, may be advantageous not only to conspecific mites, but also to other herbivores. On the contrary, plant defences induced by *T. urticae*, may negatively affect other herbivores as well, especially if the plant response is systemic. Hence, in chapter 1, I have investigated whether *T. evansi* and *T. urticae* affect induced tomato plant defences locally or systemically. As a result, *T. evansi* seems to manipulate plant defences mainly on its feeding site, because its oviposition performance was only positively affected at the site of its attack, but not in adjacent tomato leaflets. Similarly, since feeding by *T. urticae* did not confer resistance against *T. evansi* in adjacent leaflets, it suggests that the induction by *T. urticae* occurs mainly locally. Together with the dense web that *T. evansi* spins over its colonies, which has been suggested to hinders competitors (Sarmento *et al.* 2011b), we conclude that these two strategies together (the web and the suppression of plant defences by *T. evansi* only at its feeding site) can confer advantages to avoid other herbivores of profiting the suppression of plant defence. Furthermore, it also suggests that within time, *T. evansi* population would increase by profiting from the suppression of plant defence, while the amount of other herbivores would decrease in other plant parts.

CHAPTER 2: Manipulation of plant defences by *Tetranychus evansi* in bean plants

In chapter 2, I investigated whether *T. evansi* manipulate induced plant defences in common bean (*Phaseolus vulgaris*) and (ii) whether plant defence manipulation on bean plants can benefit other herbivores. I showed that the reproductive performance of *T. urticae* was higher when it shared a leaf with *T. evansi*, suggesting that suppression of plant defences by *T. evansi* benefits *T. urticae*. Since *T. evansi* was found to suppress both JA and SA pathways in tomato plants and this suppression was correlated with an increase in the oviposition performance of *T. evansi* and *T. urticae* (Sarmento *et al.*, 2011a), now I investigate whether both pathways are suppressed by *T. evansi* in bean plants. I found that *T. urticae* highly induced the accumulation of SA and the expression of the SA-related genes *PRP-2* and *PAL-1*, whereas *T. evansi* did not. However, the suppression of JA pathway only occurred in the first steps of the pathway. Furthermore, the expression of the JA-related gene *WICP* was suppressed by both spider mite species. Therefore, although here I did not clearly show that *T. evansi* manipulates the JA pathway in bean plants, it inhibited the activation of the SA pathway and this inhibition seems to be responsible for an increase in the reproductive performance of another herbivore, the *T. urticae*. Therefore, due to the similarities of our results with previous reports of suppression using tomato plants, possibly *T. evansi* ability to suppress plant defences is a widespread mechanism and might not be only employed in tomatoes or beans, but also in other plants.

CHAPTER 3: Effects of jasmonate-induced tomato defences on herbivorous mites and their predators

In Chapter 3, I further investigated how direct tomato plant defences, which can be suppressed by *T. evansi* and induced by *T. urticae*, affect the third trophic level, more specifically the predatory mite *Phytoseiulus longipes*. Here I used a defence-rescued protocol of the tomato JA-biosynthesis mutant *def-1* to first, evaluate the relationship between spider mite reproductive performance and jasmonate defences. Second, I tested to which extent these defences affected subsequently the feeding preference and intensity of predatory mites preying on the eggs of these spider mites. My data showed that the reproductive performance of both spider mite species and the predatory mite is negatively affected by JA defences. In addition, I also show that predatory mites eat more prey eggs when these came from JA-undefended plants than from JA-defended plants. It suggests that defence suppression by *T. evansi* can backfire, since it makes its eggs more vulnerable to predation and they even seem to be more palatable to predators. However, for evaluating the costs and benefits of defence suppression more research is still needed to evaluate the consequences of plant defence suppression by *T. evansi* and how suppression affects the population dynamics of predator and prey involved in this interaction.

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CHAPTER 1

Local and systemic effects of suppression of plant defences by *Tetranychus evansi* and induction by *Tetranychus urticae*

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Abstract

Plant secondary compounds are known to be produced locally and systemically after herbivore attack and are involved in plant defence. These compounds are mobilized through the vascular system and they can induce a response in cells distal to the damage. Feeding by the herbivore *Tetranychus urticae* induces a plant response, which confers resistance to tomato plants. In contrast, the red spider mite *Tetranychus evansi* is able to suppress induced defences of tomato plants, resulting in a two-fold increase of its oviposition compared to undamaged plants. However, suppressing plant defences may be advantageous not only to the red spider mites themselves, but also to other herbivores, especially when such suppression is not local, but systemic. Here, we investigated whether *T. evansi* and *T. urticae* affect induced tomato plant defences locally or systemically. To this end, we assessed the oviposition of *T. evansi* on leaflets and leaves with different levels of vascular connectivity that were previously attacked by either one of the two species. We also assessed the activity of proteinase inhibitors (PI), one of the compounds involved in direct defence in tomato, at different spatial levels. We found that the suppression of PI by *T. evansi* and induction of PI by *T. urticae* does occur beyond the feeding site. However, induction and suppression of PI decrease with increasing distance to the feeding site, suggesting that the suppression of plant defence by *T. evansi* and induction by *T. urticae* is partially, but not completely systemic. Interestingly, the oviposition of *T. evansi* was only affected on leaflets previously damaged by the spider mites *T. evansi* and *T. urticae*, but not on induced leaflets, suggesting that here PI are not the compounds responsible for a negative effect on *T. evansi* oviposition. We argue that previous feeding by spider mites *T. evansi* and *T. urticae* promotes change in leaf quality and hence, differences in the oviposition of *T. evansi*, whereas leaflets that do not suffer previous feeding do not undergo changes in quality and hence, it does not promote differences in the oviposition of *T. evansi*.

KEYWORDS – plant resistance, plant defence, systemic induction, local induction, tomato, *T. evansi*, *T. urticae.*

Introduction

Plants possess an effective resistance system, based on a combination of physical and chemical features, which plays a role in controlling damage caused by insects (Schoonhoven *et al.*, 2005). The production of plant defensive compounds constitutes on a barrier against herbivorous arthropods (Schoonhoven *et al.*, 2005; Howe & Jander, 2008) and increases the chances of plant survival and reproduction (Karban & Myers, 1989). Plants can accumulate constitutively high levels of defence compounds or mount specific defences against the attacking herbivores (Arimura *et al.*, 2005). This so-called induced response has an advantage over constitutive defence (Karban & Baldwin, 1997; Howe & Jander, 2008). For instance, the innate ability to activate direct and indirect plant defences can reduce the costs involved in defence (Hamilton *et al.*, 2001) and the plant can allocate the resources to valuable plant parts (Iwasa *et al.*, 1996; van Dam *et al.*, 1996; Bezemer *et al.*, 2003) or to use it for growth or reproduction (Arimura *et al.*, 2005).

Proteinase inhibitors (PI) are one of these plant defensive compounds, which interfere with growth and development of herbivores when present in high concentrations and play a potent defensive role against arthropods (Ryan, 1990). The PI and other plant defensive compounds are known to be produced locally (attacked site) and systemically (plant tissues adjacent to the attacked site) (Karban & Myers, 1989) through signalling molecules that are produced at the site of herbivore attack (Koo & Howe, 2012).

Systemic signals are mobilized through the vascular system and induce a wound response in cells distal to the injury (Koiwa *et al.*, 1997). Because not all leaves on a plant are connected to the same degree, the induction of defence is higher in systemic leaves that have higher vascular connections to the attacked leaf, the so-called orthostichous leaves. Leaves that are better connected are usually vertically aligned on the stem (Orians, 2000). For instance, in tomato, vascular connectivity determines the activity of proteinase inhibitors in undamaged leaflets from a damaged plant. Hence, the PI levels are highest in the damaged leaflet and lower in other leaflets of the same leaf. Considering the other leaves of the plant, the connectivity is the lowest with the leaf below the damaged leaf (Stout *et al.*, 1996a), which consequently has the lowest PI levels. Leaves with less connectivity are considered as non-orthostichous leaves and due to the low levels of defensive compounds are usually less defended against herbivores.

While plants have evolved these sophisticated mechanisms, herbivores have evolved counter-adaptations to overcome many of these mechanisms to increase their performance (Karban & Agrawal, 2002). For instance, herbivores may have adapted to sequester the defensive chemicals of the host plant for their own protection (Karban & Baldwin, 1997) or dispose of them via (enzymatic) degradation, modification or secretion (Schoonhoven *et al.*, 2005). In addition, arthropod herbivores have evolved proteinases that are largely insensitive to host plants PI, which allowed them to overcome the action of these plant defence compounds (Jongsma & Bolter, 1997). Moreover, some herbivores and pathogens have adapted to manipulate plants to their own benefit, such that establishment of induced defences is reduced or inhibited.

Kant *et al.* (2008) found a strain of *Tetranychus urticae* that did not induce the expression of genes responsible for the production of PI in wild type tomato plants. This is surprising because plant feeding by most strains of this generalist herbivore induce the transcription of these genes, which have been correlated with a reduction in the reproductive performance of spider mites on tomato plants (Li *et al.*, 2002; Ament *et al.*, 2004; Kant *et al.*, 2004). In addition, Sarmento *et al.* (2011a) found that the red spider mite *Tetranychus evansi*, a specialist on Solanaceae (Migeon *et al.*, 2009), performed much better on tomato leaves that were previously attacked by conspecifics. Since leaves from plants that were previously attacked by this mite were found to have much lower levels of PI even than leaves from unattacked plants, it is possible that the increased mite performance may be due to the plant failing to produce defensive compounds, such as the PI.

The suppression of plant defences may be advantageous not only to *T. evansi*, but also to other herbivores, as for instance the closely related spider mite *T. urticae.* Indeed, Sarmento *et al.* (2011b) found that a defence-inducing genotype of *T. urticae* doubled its fitness on leaves previously damaged by *T. evansi*. This suggests that the low activity of defensive compounds positively affects the oviposition of *T. urticae* due to the high nutritional quality of the plants. Furthermore, like many spider mites, *T. evansi* forms colonies on its host plant, which it covers with web. Sarmento *et al.* (2011b) showed that *T. evansi* is able to manipulate its feeding site by covering it with a dense web, which reduces accessibility to *T. urticae*. Hence, leaves without colonies and damage of *T. evansi* are not covered with web, and other herbivores have free access to these leaves. Consequently, the exclusion of these other herbivores by covering colonies with web is effective only when the suppression of the defence is not systemic, but restricted to those areas occupied by colonies and covered with web.

Since *T. urticae* induces tomato plant defences, including PI (Li *et al.*, 2002; Ament *et al.*, 2004; Kant *et al.*, 2004), herbivores together with *T. urticae* might suffer from the presence of these defensive compounds. Indeed, Sarmento *et al.* (2011a) has shown that *T. evansi* is sensitive to the defences induced by *T. urticae*. Hence, whereas the oviposition of *T. evansi* increased on leaves previously damaged by *T. evansi*, it decreased on leaves previously damaged by *T. urticae*. Therefore, both herbivores affect each other through induced plant responses, i. e. whereas the suppression of plant defence by *T. evansi* can positively affect the performance of spider mites, the induction of plant defence by *T. urticae* can negatively affect them.

In natural communities, suppression and induction of plant defences might have ecological consequences, especially when such mechanism is not local, but systemic throughout the plant. We therefore, investigated whether *T. evansi* is able to suppress plant defences only at its feeding site or whether this suppression also occurs systemically. Similarly, we also investigated if the induction of direct plant defences by *T. urticae* occurs locally or systemically. To do so, we measured the oviposition of *T. evansi* and the levels of PI on damaged and systemic leaflets of a plant attacked by either *T. evansi* or *T. urticae*.

Materials and Methods

Plants

Tomato seeds (*Solanum lycopersicon* var. Santa Clara I-5300) were sown once per week in trays using a commercial substrate for vegetables (Plantmax, Eucatex Agro) and kept into a controlled and herbivore-free greenhouse under controlled conditions (25±5°C and relative humidity of 70±10%). After 20 days the seedlings were transferred to plastic pots (2L) containing the same substrate and were daily watered and weekly fertilized (20:5:20 N:P:K) until the beginning of the experiments. To evaluate the systemic plant response within a leaf, we used plants with 2-4 completely developed leaves and for the systemic plant response in distal leaves, plants with 5-7 leaves. Other tomato plants of this same variety were used for spider-mite rearing.

Mite rearings

Rearings of the two phytophagous mites (*T. evansi* and *T. urticae*) were maintained in the University of Viçosa since 2002 and were obtained from naturally infested tomato plants of the same variety as mentioned above. Both mite species were reared on detached tomato leaves inside small PVC tubes with water to maintain leaf turgor. These tubes were placed inside PVC trays with a layer of water and soap to avoid contamination with other non-flying arthropods. The mass cultures were kept in a room (25±5°C; 70±10%, under a 12:12 L:D photoperiod).

Herbivory experiments

To evaluate the local and systemic plant response, we measured the oviposition of *T. evansi* and the levels of PI on damaged and systemic leaflets. Leaflets and leaves were selected according to their vascular connectivity (Stout *et al.*, 1996a; 1996b; Orians *et al.*, 2000; Orians, 2005; Frost *et al.*, 2007). Hence, the experiments were designed allowing us to assess the systemic plant response at two different spatial levels, (i) to assess the systemic plant response within a leaf and (ii) the systemic plant response in distal leaves.

Systemic plant response within a leaf

The first induction experiment was carried out using the five leaflets of a fully expanded leaf. Fifty inducer mites (*T. evansi* or *T. urticae*) were introduced on the first leaflet (A, in Fig. 1) attached to the petiole in order to induce a local or systemic response in the plant. Two days later, two leaflets (B and D, Fig. 1) were infested with three *T. evansi* mites, which served to measure oviposition on these induced leaflets.

Considering the vascular connectivity among the leaflets on a leaf, the leaflet B is more connected with the leaflet A and the leaflet D is more connected with the leaflet E (Stout *et al.*, 1996a; Orians *et al.*, 2000; Orians, 2005). Thus, we introduced spider mites on both leaflets B and D expecting to obtain a higher effect of the inducer treatment (on leaflet A) in the leaflet B and consequently to measure this effect on *T. evansi* oviposition. We used adult females of 4 days old since becoming adults. At this age, egg production is maximal and does not vary much (Bonato, 1999). Insect glue (Cola Entomologica; Bio-Controle, Sao Paulo, Brazil) was applied to the petioles of the leaflets to avoid mite movement to other plant parts.

Control plants were kept without inducer mites on the first leaflet, to avoid induction, but receiver mites were introduced as above. These plants were from the same batch and the same phenological stage as the other plants and the leaflets were also treated with glue. During the following 2 days, the number of receiver mites was assessed repeatedly and disappeared or dead mites were replaced by new ones of the same age. After these two days, the oviposition rate of the receiver mite *T. evansi* was assessed, using a stereomicroscope for egg counting. The number of eggs per female was calculated and corrected for the numbers of mites present at the various time intervals. At this time point we collected the other two remaining undamaged leaflets, C and E, and the damaged leaflet A. The leaflets were immediately frozen in liquid nitrogen and stored at -80°C until proteinase inhibitor activity analysis (see below). Hence, the three treatments were: (i) *T. urticae* (inducer on leaflet A) x *T. evansi* (receiver on leaflet B and D); (ii) *T. evansi* (inducer on leaflet A) x *T. evansi* (receiver on leaflet B and D); (iii) and control with only glue as inducer on leaflet A and *T. evansi* as receiver on leaflet B and D.

The oviposition rate of the receiver mite *T. evansi* was assessed on leaflets attached to the plants. However, using this set up, we are not sure whether 3 receiver mites are enough to promote changes in plant defence locally during feeding, which could possibly affect their own oviposition. Therefore, due to the complexity of the systemic plant response, one alternative found was to assess the oviposition rate of only one receiver mite on a detached leaflet. Hence, we now carried out a similar experiment, but instead of measuring *T. evansi* oviposition directly on the plant, we measured it on detached leaflets.

As done in the previous experiment, we infested the first leaflet (A, in Fig. 1) of a fully expanded tomato leaf and placed fifty inducer mites (*T. evansi* or *T. urticae*) in order to induce a local or systemic response in the plant. Two days later, the adjacent leaflets (B and D, Fig. 1) and the infested leaflet (A) were detached from the plant and leaf discs (diam.= 20mm) were made from these leaflets and offered to the spider mites. Sarmento *et al.* (2011a) have shown that *T. evansi* doubled its oviposition on leaves previously damaged by *T. evansi* mites. On the contrary, *T. evansi* was sensitive to the defences induced by *T. urticae* when it fed on leaves previously infested by *T. urticae*. Hence, the induction of local plant defences by *T.*

urticae and suppression by *T. evansi* can be evaluated through *T. evansi* oviposition rate. Therefore, besides evaluating the systemic effect of plant defences, we included two other control treatments to assure about the local effect of the suppression and induction.

The first control treatment was done with all the five leaflets from a leaf infested by *T. evansi*, where the mites are known to have high oviposition rates. The second treatment had leaves infested by *T. urticae*, where spider mites are known to have low oviposition rates (Sarmento *et al.*, 2011a). Thus, as well as done for the previous experiment, we kept the three treatments described above: *T. urticae* (inducer on leaflet A) x *T. evansi* (receiver on leaflet B and D), *T. evansi* (inducer on leaflet A) x *T. evansi* (receiver on leaflet B and D), and control with only glue as inducer on leaflet A and *T. evansi* as receiver on leaflet B and D and included two others: *T. urticae* (inducer on all the leaflets) x *T. evansi* (receiver on leaflet B and D), *T. evansi* (inducer on all the leaflets) x *T. evansi* (receiver on leaflet B and D). The leaf discs were kept individually in Petri dishes (diam.= 8cm) containing wet cotton wool. Here we offered each leaf disc to only one *T. evansi* female, which was allowed to feed and reproduce for two days. The number of eggs laid was assessed only on the leaf discs where the female was found, using a stereomicroscope.

Systemic plant response in distal leaves

In a subsequent experiment, we analysed the induction of plant defences on different leaves of the same plant. Different leaves of the same plant were infested with inducer and receiver mites, as shown in Fig. 2. First, the 2nd fully expanded leaf of a tomato plant was infested with 50 mites (*T. evansi* or *T. urticae*, inducers) per leaflet, i.e. among 150 to 200 mites, depending on how many leaflets there was on the 2nd fully expanded leaf of the tomato plant. Two days latter 3 receiver mites (*T. evansi*) were placed on the leaflets B and D (Fig. 2) of the 5th leaf of this same plant. We chose leaves 2 and 5 based on the study of Orians *et al.* (2000), who showed that infesting the 2nd leaf of a tomato plant results in a high induction of plant defences in the 5th leaf.

Control plants were kept without inducer mites on the second leaf, to avoid induction, but receiver mites were introduced as above. These plants were from the same batch and the same phenological stage as the other plants and the leaflets were also treated with glue. During the following 2 days, the number of receiver mites was assessed repeatedly and disappeared or dead mites were replaced by new ones of the same age. After these two days, the oviposition rate of the receiver mite *T. evansi* was assessed, using a stereomicroscope for egg counting.

The number of eggs per female was calculated, corrected for the numbers of mites present at the various time intervals. At this time point, we collected the other two remaining undamaged leaflets, C and E from the 5th leaf and the damaged leaflet A from the 2nd leaf. The leaflets were immediately frozen in liquid nitrogen and stored at -80°C until proteinase inhibitor activity analysis (see below). Hence, the three treatments were: *T. urticae* (inducer on leaf 2) x *T. evansi* (receiver on leaflet B and D of the leaf 5), *T. evansi* (inducer on leaf 2) x *T. evansi* (receiver on leaflet B and D of the leaf 5), and control with only glue as inducer on leaf 2 and *T. evansi* as receiver on leaflet B and D of the leaf 5.

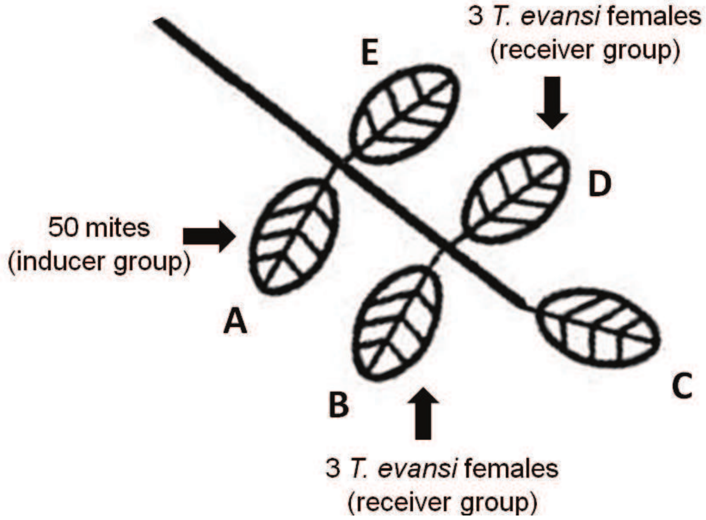

Figure 1 - Schematic drawing (adapted from Stout *et al.*, 1996a) of a fully expanded tomato leaf. The drawing shows the location of damage by the 50 inducer mites (leaflet A), the 3 receiver *T. evansi* females (leaflets B and D) and the leaflets sampled for proteinase inhibition analysis (C and E). Leaflet B has a direct vascular connection to the inducer leaflet A, leaflet D does not. The 2nd leaf from a tomato plant with 2 - 4 leaves was used in these experiments.

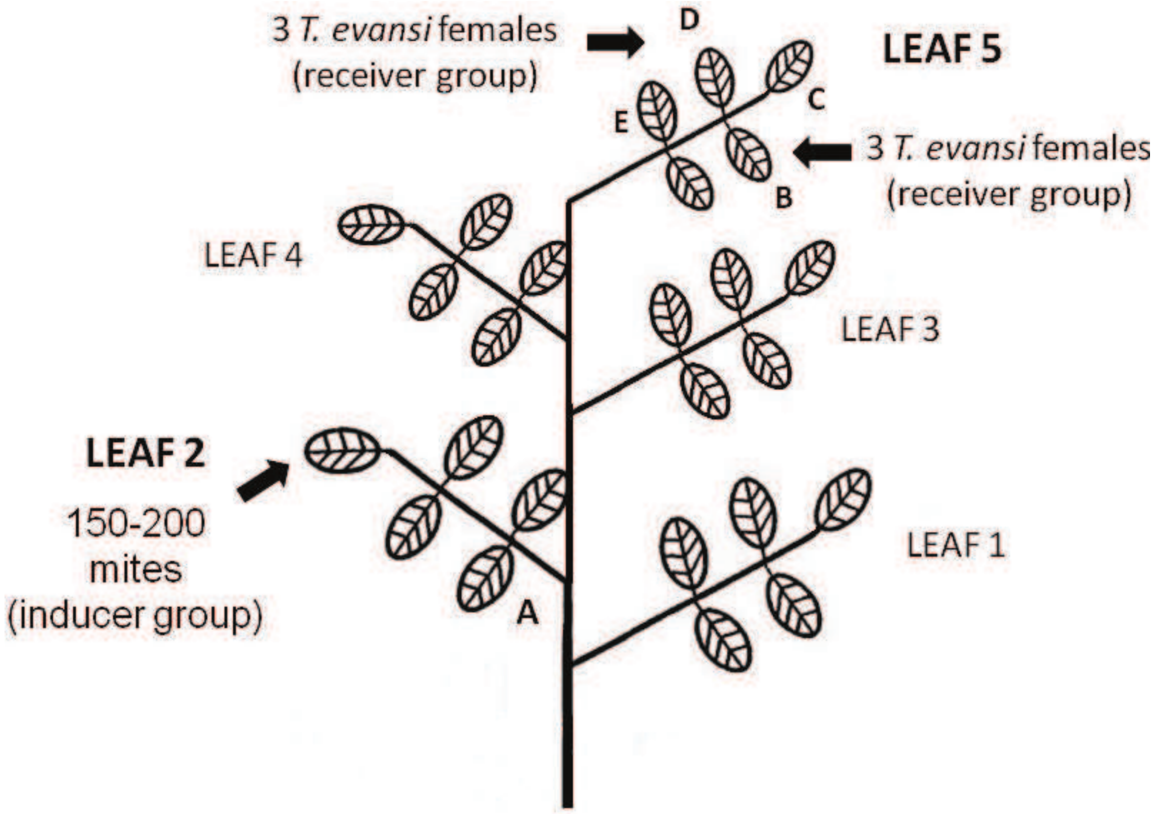

Figure 2 - Schematic drawing (adapted from *Stout et al.*, 1996a) of a tomato plant with 5 fully expanded leaves. It illustrates the location of damage by 150-200 inducer mites (2nd leaf), the 3 receivers *T. evansi* females (5th leaf, on the leaflets B and D) and the leaflets sampled for proteinase inhibition analysis (leaflets C and E of the 5th leaf). See legend to Fig. 1 for further explanation. The 2nd leaf of a tomato plant has direct vascular connection to the 5th leaf. Tomato plants with 5 - 7 leaves were used in these experiments.

Proteinase inhibitors analysis

The induction or suppression of plant defences was further verified by examining the levels of proteinase inhibitors in tomato plant leaflets. This allowed us to assess induction at three different spatial levels: local induction (leaflet under herbivory) and systemic induction (leaflet close to the infested leaflet and leaflet distal to infested leaf). Because the egg counting procedure was destructive, it was not possible to measure the proteinase inhibitor activities in the same leaflets as on which oviposition was assessed. For this reason, in the experiment which evaluates the systemic plant response within the leaf, before counting the eggs on leaflet B and D, we collected the other two remaining undamaged leaflets, C and E to evaluate the systemic induction and leaflet A, to evaluate the local induction. We joined these two leaflets to obtain enough material for the PI analysis; hence, we did not investigate the effect of connectedness of different leaflets within the same leaf. Similarly, we also collected the two corresponding undamaged leaflets from the 5th leaf (leaflet C and E) and leaflet A of the 2nd infested leaf (Fig. 2). We also carried out two controls: one with only glue applied to the petioles (referred to as “Glue”) and one without glue (“Control”). This was done to assess whether glue alone would induce PI activity.

The collected leaflets were frozen in liquid nitrogen and stored at -80C. Subsequently, the leaflets were ground to powder in liquid nitrogen and homogenized in a extraction buffer (0.1 M Tris-HCl buffer, pH 8.2 and 20 mM CaCl2; 1 : 3 w/v), centrifuged at 10.500*g* (25 min, 4C). The resulting supernatant was used for determining the trypsin and protein content. Thus, 100 µL trypsin (4.7 x 10 M) was mixed with 100 µL of the supernatant and 500 µL extraction buffer (0.1 M Tris-HCl buffer, pH 8.2 and 20 mM CaCl2), and was incubated at room temperature for 5 min.

Controls consisted of 500 µL extraction buffer and 100 µL of trypsin (4.7 x 10 M), without the supernatant from plant extract. Subsequently, the samples were mixed with 500 µL extraction buffer and 500 µL of the substrate L-BAPNA (N-α-benzoyl-L-Arg-p-nitroanilide; substrate for trypsin-like enzymes). Tripsin activity was measured with a spectrophotometer (absorbance at 410 nm), and the difference between the absorbance measured at 150 and 60 s was used to determine trypsin activity. Measurements were performed 6 times per sample, and were converted to milligram of trypsin inhibited per gram of protein and were corrected for the dilution (Kakade *et al.*, 1974). Protein concentration was measured following Bradford (1976) using solutions of 0-0.2 mg/ml of bovine serum albumin (BSA) as standards. The activity was measured with a spectrophotometer at 535 nm.

Statistical analysis

Herbivory experiments - The data from the experiments using leaflets attached to the plant were analysed using generalized linear mixed-effects models (lmer) with normal error distribution and including replicate, date and plant as random factors. The oviposition rate of *T. evansi* on various leaflets was included as response variable and treatments ("Glue", "*T. evansi*" and "*T. urticae*") and leaflets (B and D) as explanatory variable. For the experiment that assessed the *systemic plant response within a leaf* were carried out 20 replicates per treatment and for the experiment *systemic plant response in distal leaves* were carried out 17 replicates per treatment.

The data from the experiment using detached leaflets was analysed using generalized linear mixed-effects models (lmer) with poisson error distribution and including replicate, date and plant as random factors. The oviposition rate of *T. evansi* on various leaflets was included as response variable and treatments ("Glue", "*T. evansi*", "*T. urticae*", "*T. evansi* infested" and "*T. urticae* infested") and leaflets (B and D) as explanatory variable. For each treatment were carried out 20 to 23 replicates.

Proteinase inhibitors analysis - Square root-transformed proteinase inhibitor activities were analysed using a linear mixed-effects model (nlme), including replicate as random factor to correct for the 6 repeated measures done per sample. The treatments "Glue", "Control", "*T. evansi*" and "*T. urticae*" were included in the model as explanatory variable and the oviposition of *T. evansi* as response variable. Data of the inducer leaflets (A) was analysed separately from the data of the induced leaflets (C and E). Three replicates were carried out per treatment.

For all the experiments, the statistical analyses were done building complete models, including all factors and were checked with residual analyses to correct for overdispersion and distribution. In case of finding statistical differences, new models were built separately to test the effect of the treatments on the oviposition of *T. evansi*. The last two treatments were included only in the experiment with detached leaflets detailed above.

Subsequent model simplification was achieved by extracting non-significant interactions and terms ($p>0.05$) from the models, starting with higher-order interactions (backward method, Crawley, 2007). When two non-significant terms were of the same order, the one explaining least deviance was extracted first. The deletion of each term was followed by an ANOVA, in order to recalculate the deviance explained by remaining terms. Hence, new models were built and tested and when the models did not differ significantly, the term was deleted from the model. All statistical analyses were done in R statistical software, version 2.15.1 (R Development Core Team, 2010).

Results

Herbivory experiments

Systemic plant response within a leaf

In the experiment using leaflets attached to the plant, the oviposition rate of *T. evansi* was not significantly affected, either by the treatments “Glue”, “*T. evansi*” and “*T. urticae*” (lmer with $Chi_{[2,7]}$= 4.6; P= 0.10; Fig. 3) or by the leaflet position, i.e. *T. evansi* oviposition was not different on the connected leaflets B and the unconnected leaflets D (lmer with $Chi_{[1,8]}$= 1.5; P= 0.22).

In the experiment using detached leaflets, only on the infested leaflet A there was a significant effect of the treatment on the oviposition of *T. evansi* (lmer with $Chi_{[4,7]}$= 14.0; P= 0.007; Fig. 4). The oviposition of this spider mite was similar on leaflets previously infested with conspecific mites (“*T. evansi*” and “*T. evansi* infested” treatments) and on non-infested leaflets (“Glue” treatment; lmer with $Chi_{[1,4]}$= 0.3; P= 0.57) and lower on leaflets previously infested with heterospecific mites (“*T. urticae*” and “*T. urticae* infested” treatments) (lmer with $Chi_{[1,3]}$= 15.5; P≤ 0.001).

In the adjacent leaflets B and D, induced (not damaged) leaflets where the systemic effect of the three treatments (“*T. evansi*” “*T. urticae*” and “Glue”) on *T. evansi* oviposition was evaluated, there was no significant effect of the treatment on its oviposition (lmer with $Chi_{[2,4]}$= 0.2; P= 0.92). In addition, the oviposition of *T. evansi* was not affected by the leaflet position, i. e. *T. evansi* laid similar amount of eggs on leaflet B and D (lmer with $Chi_{[1,5]}$= 0.9; P= 0.35). However, on leaflets from the other two control treatments included “*T. evansi* infested” and “*T. urticae* infested” *T. evansi* clearly laid more eggs on leaflets previously infested by *T. evansi* than by *T. urticae* (lmer with $Chi_{[1,7]}$= 16.6; P≤ 0.001 for leaflet B and $Chi_{[1,7]}$= 8.4; P= 0.003 for leaflet D).

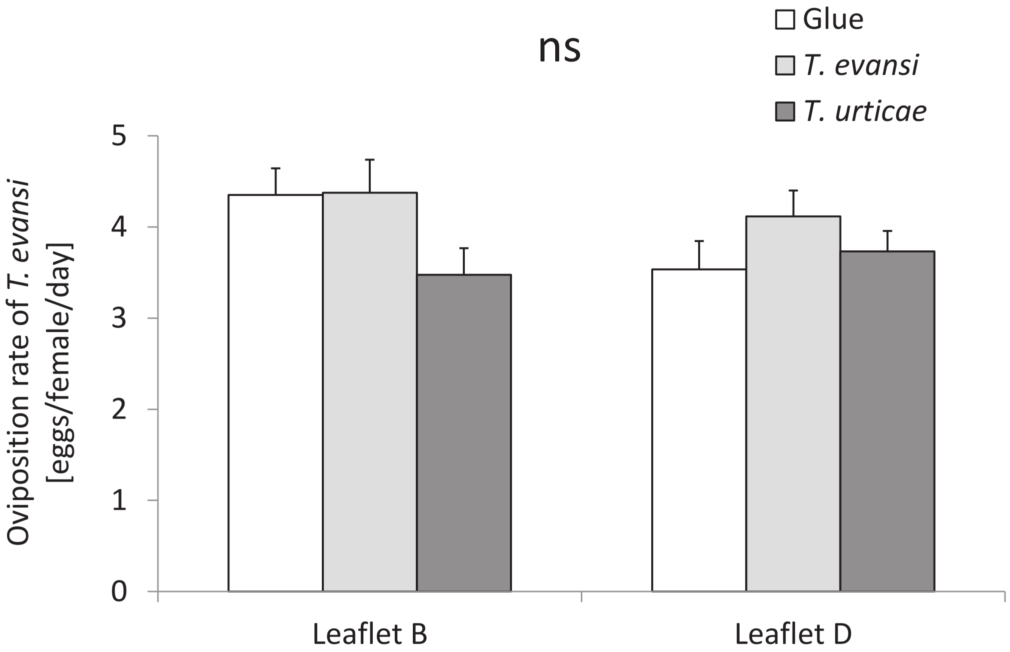

Figure 3 – Oviposition of *T. evansi* on induced leaflets close to the damaged leaflet. This experiment was done with leaflets attached to the plant. Shown is the average number of eggs (±s.e.m.) produced per *T. evansi* mites per day on the induced leaflets B (white bars) and leaflets D (gray bars) of a leaf of which one other leaflet (A, Fig. 1) was under attack by *T. evansi*, by *T. urticae*, or was unattacked (Glue); ns indicates non significant differences among the treatments and leaflets ($P > 0.05$).

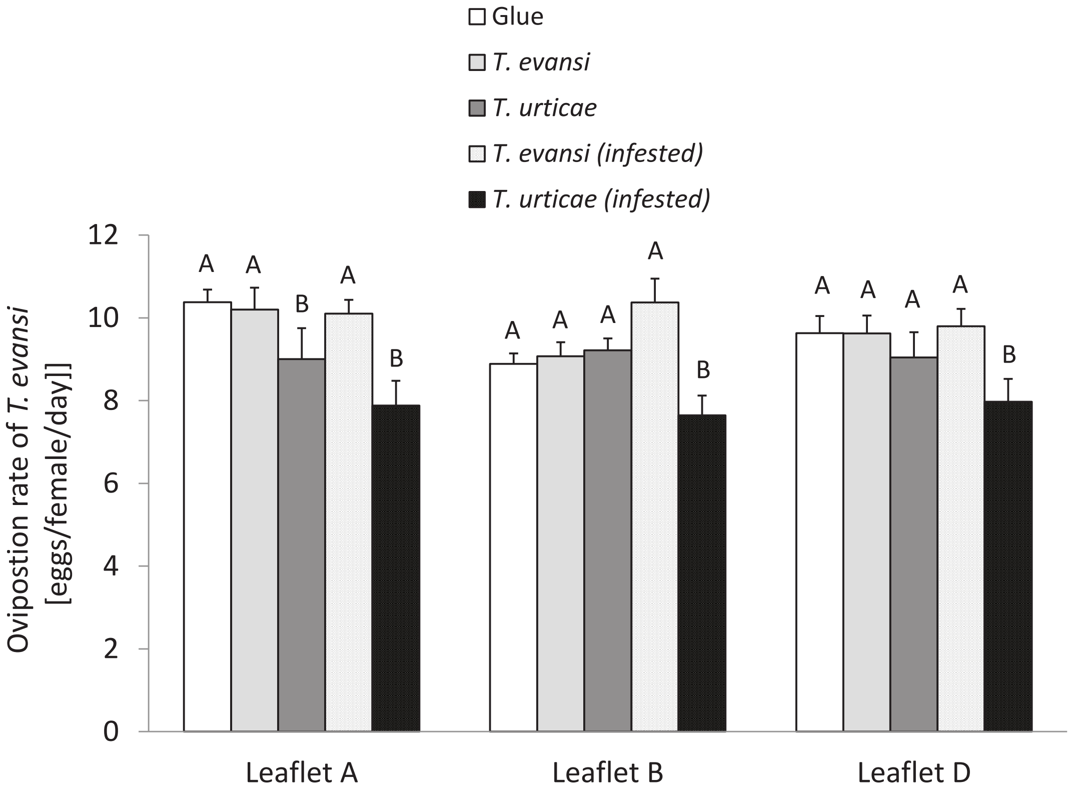

Figure 4 – Oviposition of *T. evansi* on induced leaflets close to the damaged leaflet. This experiment was done with leaflets detached from the plant; i. e. oviposition of *T. evansi* was measured on leaf discs. Shown is the average number of eggs (±s.e.m.) produced per *T. evansi* mites per day on damaged leaflets (A) and induced leaflets (B and D, Fig. 1) among the treatments "Glue", "*T. evansi*" and "*T. urticae*" and the other two control treatments included, the "*T. evansi* infested" and "*T. urticae* infested". Bars with different letters within each leaflet differ significantly.

The activity of PI found in infested leaflets (leaflet A, Fig. 1) did not differ significantly among the treatments “Control”, “Glue”, “*T. evansi*” and “*T. urticae*” (lme with Sqrt likelihood ratio (L-ratio)= 5.9; *df*= 3; P= 0.11; Fig. 5a). Although not significant, there is a trend in finding higher proteinase inhibitor activity in leaflets attacked by *T. urticae* than under *T. evansi* attack and in undamaged leaflets (“Glue” treatment, with only glue applied to their petioles and “Control”, no glue and no damage). Our results also show that the application of the glue on petioles of these inducer leaflets did not induce local plant response; PI activity was similar on plants with glue and clean plants (lme with L-ratio = 0.1; *df*= 1; P= 0.81; Fig. 5a).

The activity of PI in induced leaflets (leaflets C and E, Fig. 1) did vary among the treatments, indicating that there was a significant systemic effect of the treatments “Glue”, “*T. evansi*” and “*T. urticae*” on PI activity (lme with L-ratio = 7.0; *df*= 1; P= 0.02; Fig. 5b). It also shows that induced leaflets from clean plants with glue and plants under attack by *T. evansi* did not differ significantly (lme with L-ratio = 0.1; *df*= 1; P= 0.95), but induced leaflets of plants attacked by *T. urticae* showed higher PI activity (lme with L-ratio = 7.0; *df*= 1; P= 0.008). Our results also show that the application of the glue on petioles of these inducer leaflet A did not induce a systemic plant response in the leaflets C and E, since the PI activity was similar on plants with glue and clean plants (lme with L-ratio = 0.1; *df*= 1; P= 0.70; Fig. 5b).

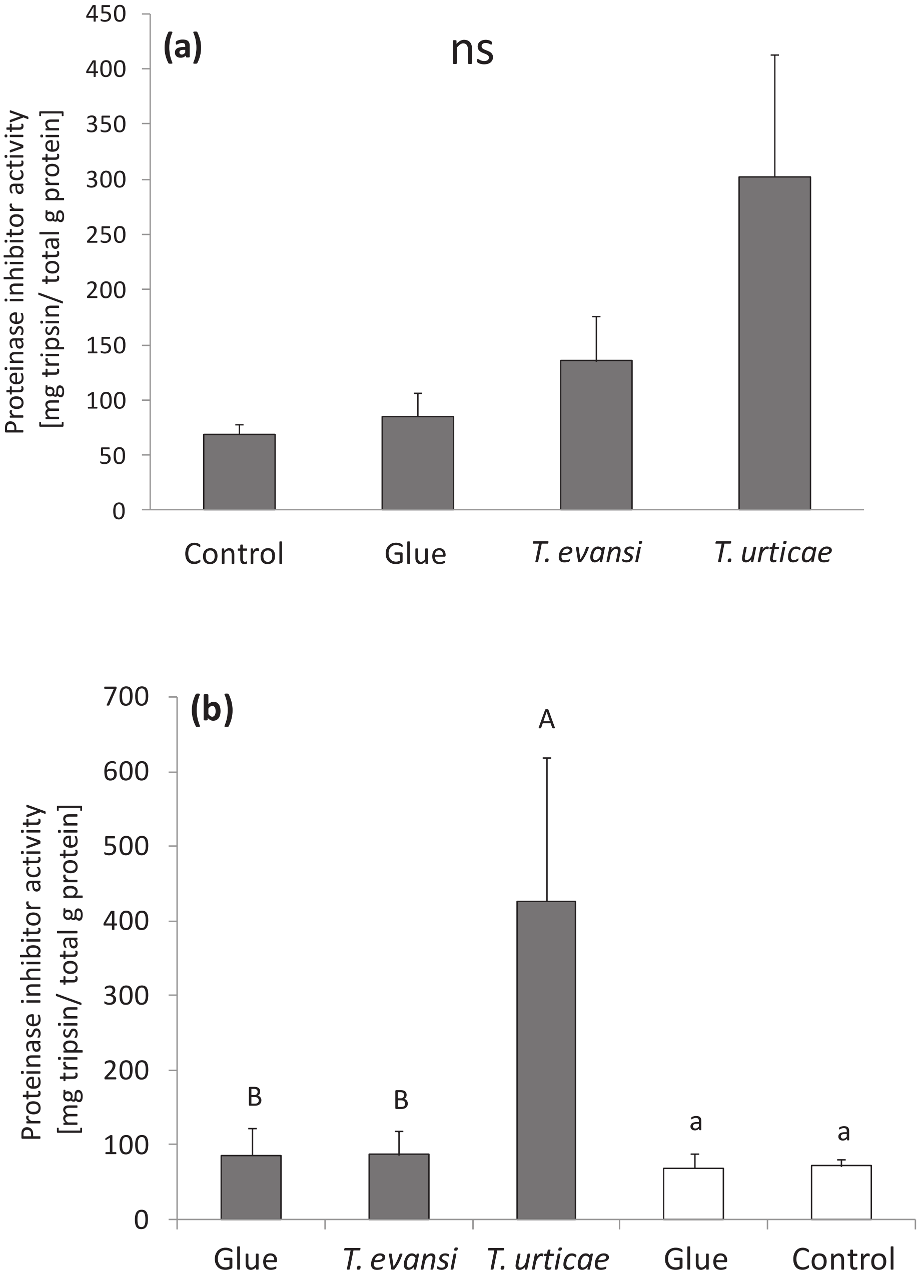

Figure 5 - Average proteinase inhibitor activity (± s.e.m., in Mg trypsin inhibited/g protein) in infested and induced tomato leaflets. (a) The tomato leaflet (A, Fig. 1) was either infested with *T. urticae*, *T. evansi,* undamaged ("Control") or undamaged, but with glue on their petiole ("Glue") and on day 5, they were sampled for measurement of local PI activity; ns indicates non significant differences among the treatments (P> 0.05). **(b)** The induced tomato leaflets (C

and E, see Fig. 1) from plants with the leaflet (A) of the same leaf attacked by *T. evansi* or *T. urticae* or undamaged plant with glue (Glue), were sampled for measurement of systemic PI activity (gray bars). On the right are shown the values of the induced leaflets (C and E, see Fig. 1) for two control treatments (white bars): the undamaged (“Control”) and the clean plant with glue (“Glue”). Different capital letters represent significant differences ($P \leq 0.05$) on the PI activity among the three treatments: *T. evansi*, *T. urticae* and Glue (gray bars) and similar small letters represent non significant differences ($P > 0.05$) on the PI activity among the treatments Control and Glue (white bars); ns indicates non significant differences among the treatments and leaflets.

Systemic plant response in distal leaves

The oviposition of *T. evansi* was not affected either by the treatments ("Glue", *"T. evansi"* and *"T. urticae"*) (lmer with $Chi_{[2,5]}$=0.8; P=0.68) or by the leaflet position (lmer with $Chi_{[1,6]}$=1.5; P=0.21, Fig. 6). It means that *T. evansi* oviposition was not different on the connected or unconnected leaflets (leaflet B and D of the 5th leaf, Fig. 2) distal to the damaged leaf (2nd leaf of a tomato plant, Fig. 2), which was attacked by *T. evansi*, *T. urticae* or unattacked (Glue).

The proteinase inhibitor activity in the damaged leaflets (leaflet A, Fig. 2), showed the same trends in the previous experiment (cf. Fig. 5a and 7a), which were again not significant (lme with L-ratio = 4.2; *df*= 3; P= 0.24; Fig. 7a). However, following the same reasoning of the first induction experiment the trends were higher on leaflets under *T. urticae* attack than under *T. evansi* attack or the two control leaflets ("Glue" and "Control"). It also shows that using just glue applied to the petioles on the inducer leaflets, is not enough to induce local plant defence, because the PI activity was similar in both controls (lme with L-ratio= 0.1; *df*= 1; P= 0.80).

The activity of PI in induced leaflets (leaflets C and E, Fig. 2) did not vary among the treatments "Glue", "*T. evansi*" and "*T. urticae*" (lme with L-ratio = 4.3; *df*= 2; P= 0.11), indicating that there was no systemic effect on the induced leaflets of the 5th leaf caused by the presence of the attacker on the 2nd leaf. Our results also show that the application of the glue on petioles of these inducer leaflet A of the leaf 2 did not induce a systemic plant response in the leaflets C and E of the leaf 5, since the PI activity was similar on plants with glue and clean plants (lme with L-ratio = 1.8; *df*= 1; P= 0.19; Fig. 5b).

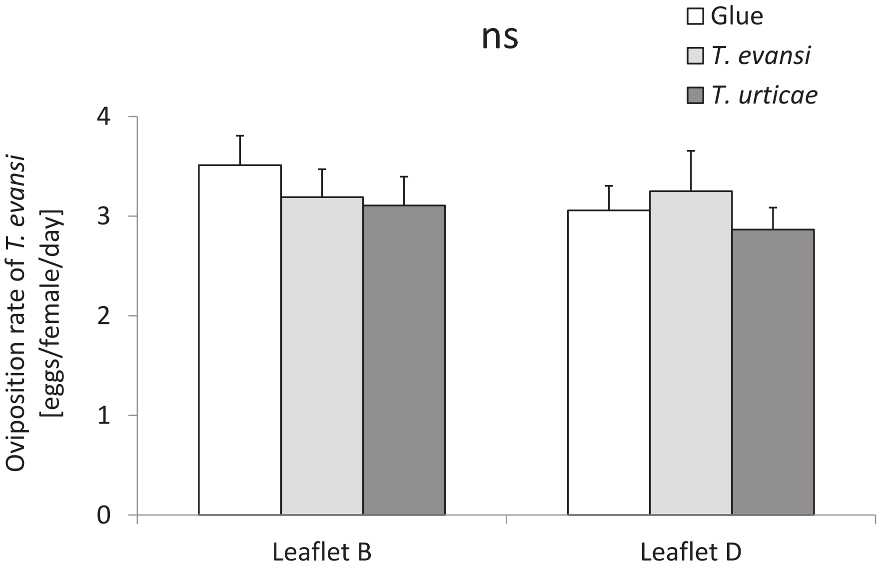

Figure 6 - Oviposition of *T. evansi* on induced leaflets distal to the damaged leaf. This experiment was done with leaflets attached to the plant. The average number of eggs (±s.e.m.) produced per each receiver mite *T. evansi* per day on undamaged leaflets B (white bars) and leaflets D (gray bars) from a 5th leaf of which another leaf (2nd leaf of a tomato plant, Fig. 2) was under attack by *T. evansi*, by *T. urticae*, or was unattacked (Glue); ns indicates non significant differences among the treatments and leaflets ($P > 0.05$).

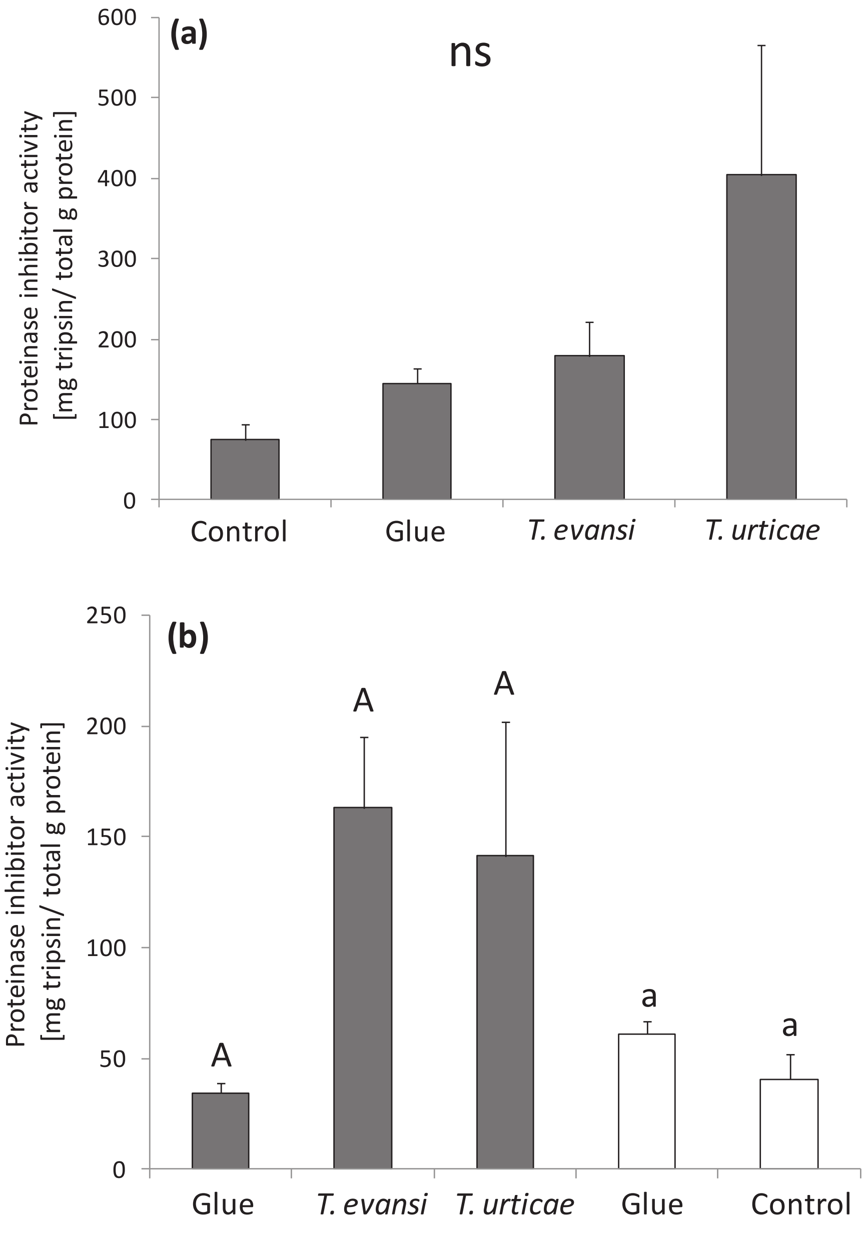

Figure 7 - Average proteinase inhibitor activity (± s.e.m., in Mg trypsin inhibited/g protein) in infested and induced tomato leaflets. (a) The 2nd leaf o tomato plant was either infested with *T. urticae*, *T. evansi,* undamaged ("Control") or clean leaflets with glue on their petiole ("Glue") and on day 5, the leaflet A (Fig. 2) was sampled for measurement of local PI activity. The effect of treatment on the activity was not significant (lme with L-ratio= 4.2; *df*= 3; P= 0.24); ns indicates non

significant differences among the treatments (P> 0.05). **(b)** The induced tomato leaflets (C and E, Fig. 2) from the 5th leaf of a tomato plant, which another leaf (2nd leaf of a tomato plant, Fig. 2) was under attack by *T. evansi* or *T. urticae* or unattacked (Glue) was sampled for measurement of systemic PI activity. On the right are shown the values of the induced leaflets (C and E from the 5th leaf, Fig. 2) for two control treatments (white bars): the undamaged (“Control”) and the clean plant with glue (“Glue”). Different capital letters represent significant differences (P≤ 0.05) on the PI activity among the three treatments: *T. evansi*, *T. urticae* and Glue (gray bars) and similar small letters represent non significant differences (P> 0.05) on the PI activity among the treatments Control and Glue (white bars); ns indicates non significant differences among the treatments and leaflets (P> 0.05)

Discussion

Our results for proteinase inhibitor activity and the oviposition of *T. evansi* assessed whether the induction or suppression of plant defences occurs locally or systemically through the plant. First, our results indicated that the PI induction by *T. urticae* and suppression by *T. evansi* occurred systemically within a leaf close to the damaged leaflet, but not in leaves distal to the damaged leaf. However, the oviposition of *T. evansi* was only influenced on damaged leaflets showing that the systemic effect of the proteinase inhibitor on *T. evansi* performance was insignificant; independently whether the leaflets were near (within a leaf) or distal (other leaves) to the damage leaflet.

Studies have shown that the induced defence in tomato foliage varies with the position of the leaf relative to the damage (Stout *et al.*, 1996a; 1996b; Orians *et al.*, 2000). Our results pointed out the induced (systemic) leaflets near connected to the damage leaflet (systemic plant response within a leaf, Fig. 5b) showed variation in PI activity among the treatments, while induced leaves distal to the damaged leaf (systemic plant response in distal leaves, Fig. 7b) did not. This result suggests that the suppression of plant defence wanes as the distance to the feeding site increases, i. e. suppression of plant defence by this mite might be only partially systemic. Hence, induction is not necessarily uniform, but varies in strength at different positions relative to damage (Stout *et al.*, 1996a). In addition, it is also important to highlight that the PI activity in leaflets distal to the leaflet damaged by *T. urticae* was lower than in leaflets close to the damaged leaflet, which would suggest that plant defence induction by *T. urticae*, as well as found for *T. evansi*, is only partially systemic.

In contrast to the results of Sarmento *et al.* (2011a), we did not find significant differences in the PI activity in infested leaflets (A) for both experiments evaluating the systemic plant response (Fig. 5a and 7a), but the observed trends were similar:

T. urticae causes increased PI activity and *T. evansi* does not. Reasons for our results not being significant as those of Sarmento *et al.* (2011a) might be that we used younger plants and a shorter period of mite infestation. Moreover, the trends observed here might indicate significance if more replicates would be done. To test this, we analysed the data of the PI activity in the infested leaflets (A) from both experiments together.

The only difference among the experiments was that in the first one (systemic plant response within a leaf), the infested leaflet (A) was near the leaflet with receiver mites (B and D), whereas in the second one (systemic plant response in distal leaves) the receiver mites were on a different leaf. However, because we released 50 (Fig. 1) or 150-200 (Fig. 2) inducer mites two days before we add the receivers, we infer that the induction in both experiments was mainly due to the effect of the inducer on leaflet A or on the 2nd leaf, respectively.

Joining the data from both experiments we found the significant effect of the treatment on the PI activity (lme with L-ratio= 8.85; *df*=3; P= 0.03). As we expected, the PI activity for the two Controls (“Control” and “Glue”) and “*T. evansi”* were lower than for *T. urticae* treatment (lme, L-ratio= 7.13; *df*=1; P= 0.007). Hence, with these results we can infer that more replicates would significantly change our results in agreement with those previously found by Sarmento *et al.* (2011a), where leaflets attacked by *T. urticae* induces the activity of PI whereas leaflets attacked by *T. evansi* does not.

The oviposition of *T. evansi* was only significantly affected on the previously infested leaflet A (Fig. 3). The oviposition of this spider mite was higher on leaflets previously damaged by *T. evansi* than on leaflets previously infested with *T. urticae*. It suggests that feeding by spider mites promoted changes in the leaf quality and it consequently affected the oviposition of *T. evansi*. These results are in agreement with Sarmento *et al.* (2011a) and Sarmento *et al.* (2011b), which showed that leaflets previously damaged by *T. evansi* promotes the oviposition of con-specifics, whereas leaflets previously damaged by *T. urticae* negatively affected the oviposition of *T. evansi*.

We found no effect of the systemic plant response in the oviposition of *T. evansi* within a leaf or in leaves distal to the damaged leaf. The non significant systemic effects of the treatments and the position of the leaflets on *T. evansi* oviposition suggest that although there are differences on the systemic induction of PI within a leaf, this systemic effect did not affect *T. evansi* oviposition.

Our results from the experiment evaluating the systemic plant response within a leaf using detached leaflets supported this assumption (Fig. 4), because as well as found for this same experiment using attached leaflets, the oviposition of *T. evansi* was not affected either by the leaflet position or by the treatments. However, when *T. evansi* was placed on the leaflets B and D previously infested by *T. evansi* and by *T. urticae* ("*T. evansi* infested" and "*T. urticae* infested", Fig. 4) its oviposition of was clearly higher on leaflets previously infested by *T. evansi* than on leaflets previously infested by *T. urticae*. Therefore, the addition of these controls was important to show that indeed the local effect of the suppression by *T. evansi* can increase the quality of the leaflets and the oviposition of the spider mites, but that there was no significant systemic effect of the suppression.

Similarly, since feeding by *T. urticae* did not confer resistance against *T. evansi* in adjacent leaflets, it suggests that there is no systemic effect of the induction by *T. urticae*. This result differs from what has been shown by Karban & Carey (1984), which reported that induced defence by prior herbivory of *T. urticae* or *T. turkestani* in cotton seedlings reduced the population growth of *T. urticae*.

It is surprisingly that *T. evansi* did not oviposit differently on the leaflets B and D on leaves infested with conspecifics mites (*"T. evansi"* treatment) or heterospecifics mites (*"T. urticae"* treatment). Considering that the leaflet B is more connected with the leaflet A (Stout *et al.*,1996a; Orians *et al.*, 2000; Orians 2005) and that *T. evansi* suppresses plant defences and suppression correlates with a positive effect on the oviposition rate of conspecifics (Sarmento *et al.* 2011a), it was expected to find a higher oviposition on leaflets B than on leaflets D on plants under its attack. On the opposite, because *T. urticae* is known to induce plant defence (Li *et al.*, 2002; Ament *et al.*, 2004; Kant *et al.*, 2004), it was expected to find more eggs on the leaflet D, which is less connected with the leaflet A. In this case, the leaflet D is expected to contain smaller amounts of defensive compounds and consequently is where mites are supposed to oviposit more.

As we inferred that the suppression of PI by *T. evansi* is not uniform, an increase in oviposition of at least in the experiment evaluating the systemic effect of the suppression within a leaf would be expected. On the opposite, as the distance from the damaged leaflet increases as a consequence, we expect that the positive effect of the suppression of plant defence on herbivorous performance decreases. An explanation for these results is that does not necessarily exist a linear correlation among the PI activity and the oviposition of spider mites (Kant *et al.*, 2008; Sarmento *et al.*, 2011).

We need also to consider that the oviposition rate is an ecological measure, which is, therefore, not only affected by the presence of PI in the leaf but also by a multitude of taste stimuli offered by the plant (Schoonhoven *et al.*, 2005) and biotic

(Chen *et al.*, 2008) and abiotic factors (Fink & Völkl, 1995). The balance of stimulatory and inhibitory compounds will determine the final acceptance to oviposit or not on the leaf plant surface.

In summary, our results showed that suppression by *T. evansi* positively affect the performance of the conspecifics at the site of its attack, but not in adjacent leaflets. We argue that feeding by spider mites promotes change in leaf quality and hence, differences in the oviposition of *T. evansi*, whereas leaflets that do not suffer previous feeding by *T. evansi* mites do not undergo changes in quality and hence it does not promote differences in the oviposition performance.

Together with the dense web that *T. evansi* spins over its colonies, which hinders competitors (Sarmento *et al.* 2011b), this limits the possibilities of other herbivores to profit from the suppression. Furthermore, because we inferred that *T. evansi* is able to manipulate plant defence mainly on its feeding site we suggest that through time its population would increase by profiting from the suppression of plant defence, while the amount of other herbivores would decrease in other plant parts. This difference can be remarkable as soon as *T. evansi* grows and overexploit the plant, eliminating *T. urticae* within weeks (Sarmento *et al.,* 2011b).

We conclude that these two strategies together (the web and the suppression of plant defences by *T. evansi* at its feeding site) can confer advantages to avoid other herbivores from profiting the suppression of plant defence. Nevertheless, this work provides an insight about how suppression occurs beyond its feeding site.

Acknowledgements

We thank CAPES, CNPQ and FAPEMIG for financial support.

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CHAPTER 2

Manipulation of plant defences by *Tetranychus evansi* in bean plants

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Abstract

When plants are attacked by herbivores they establish a defence response. This defence response is characterized by activation of signalling pathways such as the jasmonic acid (JA) and salicylic acid (SA) pathways. The generalist spider mite *Tetranychus urticae* induces SA and JA pathways in tomato plants and especially the induction of JA has been suggested as responsible for lowering their reproductive performance on induced plants. In contrast, the spider mite *Tetranychus evansi,* which is a specialist of Solanaceae, was found to suppress both JA and SA pathways in tomato plants and this suppression was correlated with an increase in the oviposition performance of *T. evansi* and *T. urticae*. Here we investigated (i) whether this suppressor mite is able to manipulate the JA and SA pathway in another plant, the common bean (*Phaseolus vulgaris*) and (ii) whether plant defence manipulation on bean plants can benefit other herbivores such as *T. urticae*. To do so, bean leaves were infested or co-infested with both spider mite species or left clean, and phytohormone accumulation and relative expression of genes regulated by JA and SA were quantified. We found that *T. urticae* highly induced the accumulation of the phytohormone SA and the expression of the SA-related *genes PRP-2* and *PAL-1*, whereas *T. evansi* did not. The signalling molecules traumatic acid (TA) and 12-oxo-phytodienoic acid (OPDA) from the JA pathway were induced by *T. urticae*, but were not induced by *T. evansi*. However, the accumulation of the phytohormone JA was induced by both spider mite species and the jasmonoyl-isoleucine (JA-Ileu), the main JA bioactive compound, was not significantly affected by spider mite attack, either by *T. urticae* or by *T. evansi*. Similarly, the expression of the gene *WICP* (JA-related) was not significantly affected by spider mite attack. Summarizing, *T. evansi* seems to manipulate SA pathway, but the manipulation of JA pathway needs to be investigated in more details. Furthermore, the reproductive performance of *T. urticae* was higher when it shared a leaf with *T. evansi*, suggesting that suppression of plant defences by *T. evansi* benefits *T. urticae*. Finally, here we show that *T. evansi* manipulates the induction of plant defences in bean plants. Due to the similarities of our results with previous reports of suppression using tomato plants, possibly, *T. evansi* ability to suppress plant defences is a widespread mechanism and might be employed in other plants besides tomato and beans. In addition, suppression of tomato defences might not be the prime reason for the specialism of *T. evansi* on Solanaceae.

KEYWORDS – plant resistance, plant susceptibility, beans, plant defence, *T. evansi*, *T. urticae.*

Introduction

Plants are often attacked by herbivores and produce compounds to reduce the performance of these attackers (Karban & Myers, 1989; Karban & Baldwin, 1997). Such direct plant defences render plants more resistant to subsequent herbivory and can be displayed constitutively, i. e. be always present in a plant, or be induced after herbivore attack (Karban & Baldwin, 1997; Arimura *et al.*, 2005). A central step to promote the induction of plant defences is the activation of signalling cascades such as salicylic acid (SA) and jasmonic acid (JA) pathways, the two main signal transduction routes involved in plant defences. These pathways can act separately or interact via cross-talk mechanisms (Koornneef & Pieterse, 2008).

Traditionally, it was thought that the SA pathway plays a role in promoting resistance against biotrophic pathogens (Robert-Seilaniantz *et al.*, 2011) by inducing the production of pathogenesis-related proteins (PRPs), which are involved in the systemic acquired resistance (SAR) (Ryals *et al.*, 1996). SA has been identified as an endogenous phloem-mobile compound (Yalpani *et al.*, 1991) and its role in plant resistance has been demonstrated in several plants, such as tobacco (Malamy *et al.*, 1990), cucumber (Métraux *et al.*, 1990; Molders *et al.*, 1996), rice (Silverman *et al.*, 1995), beans (Clarke *et al.*, 1998) and *Arabidopsis* (Lawton *et al.*, 1995).

How SA is synthesized in plants is not fully understood, but one way is to synthesize SA from cinnamate, regulated by the activity of phenylalanine ammonia lyase (*PAL*) genes. The *PAL* gene expression is responsive to a variety of environmental stimuli, such as plant growth, development, stress conditions (Huang *et al.*, 2010) and pathogen infection (Mauch-Mani & Slusarenko, 1996; Pallas *et al.*, 1996).

It was also traditionally believed that the JA pathway acts mainly as defence against chewing insects, cell content feeders (Walling, 2000) and necrotrophic pathogens (Robert-Seilaniantz *et al.*, 2011). The activation of JA pathway (also called as octadecanoid or lipoxygenase pathway) has been characterized in detail, in particular with respect to its role in the regulation of plant defence responses. The best known jasmonates are jasmonic acid (JA), its precursor, 12-oxo-phytodienoic acid (OPDA) and jasmonoyl-isoleucine (JA-Ileu), which appeared as the major bioactive form of JA and is related with the expression of typical defence genes (Li *et al.*, 2002; Koo & Howe, 2012), leading to the biosynthesis of defensive proteins, such as proteinase inhibitors (Green & Ryan, 1972) and polyphenol oxidases (PPOs) (Constabel *et al.*, 1995). Tomato and other solanaceous plants have been used as a model system to study the JA pathway since Green & Ryan (1972) reported the accumulation of proteinase inhibitors (PIs) in wounded tomato leaves.

Nowadays, it is well known that JA defences are employed by a wide range of plants and the JA pathway has also been extensively studied in *Arabidopsis thaliana* (McConn *et al.*, 1997; Glauser *et al.*, 2008; Gfeller *et al.*, 2011), especially to investigate its importance during pathogen infection (Demianski *et al.*, 2012; Ren *et al.*, 2012).

It is also known that some variations in the proposed JA pathway can occur among plants. For instance, studies in *Arabidopsis* present a more complex view of the JA pathway than that proposed for solanaceous species. In tomato plants, oligosaccharides, JA and ethylene participate in the expression of PIs, while in *Arabidopsis*, oligosaccharides repress JA-related genes and activate a set of JA-independent genes (Rojo *et al.*, 1999). In contrast to tomato, JA and ethylene did not act in concert for the expression of JA-related genes. Therefore, although wide spread among plant kingdom different plants may employ distinct mechanisms to regulate JA synthesis in response to damage or attack.

Whereas JA pathway is believed to act mainly against herbivores and SA against pathogens, plant defences against spider mites are characterized by simultaneous activation of both SA and JA pathways (Kant *et al.*, 2004). In tomato plants, the down-regulation of especially the JA defences coincides with a increase in spider mite performance (Sarmento *et al.*, 2011a), while the up-regulation coincides with lower reproductive performance of spider mite (Li *et al.*, 2002; Ament *et al.*, 2004; Kant *et al.*, 2004). Most genotypes of the spider mite *Tetranychus urticae* (Acari: Tetranychidae) induces the transcription of JA-related defence genes, such as the wound-induced proteinase inhibitor I (*WIPI-I*) and II (*WIPI-II*) and SA-regulated genes such as the pathogenesis-related protein 6 (*PRP-6*) (Ament *et al.*, 2004; Kant *et al.*, 2004).

Recently, some spider mite strains and species are shown not to induce or even to suppress induced plant defences. For instance, Kant *et al.* (2004) found a line of *T. urticae* that did not induce JA-related direct tomato defences and Sarmento *et al.* (2011a) found a spider mite species, *Tetranychus evansi* that was able to suppress SA and JA tomato plant defences. *T. evansi*-infested tomato plants were found to have suppressed transcript levels and enzymatic activities of PIs, and contained lower levels of PI activity even than unattacked plants. In addition, not only the gene *WIPI-II*, which is regulated by JA pathway, but also the *PRP-6* (SA-related) was not induced by *T. evansi* attack. This suppression was found to promote not only an increase in the reproductive performance of *T. evansi*, but also increased the reproductive performance of its competitor *T. urticae* (Sarmento *et al.*, 2011b). Hence, *T. evansi* has been indicated as a suppressor of JA and SA defences in tomato plants.

Considering that JA and SA plant defences are employed by a wide range of plants and that *T. evansi* is able to overcome these defences in tomato plants, we believe that its ability to suppress JA and SA defences is a widespread feature among plants and is not only restricted to tomato plants. Since *T. evansi* is a specialist of Solanaceae (Migeon *et al.*, 2009), but it feeds and reproduces on bean leaves under laboratory conditions (Ataide LMS, personal observation) here we chose the common bean (*Phaseolus vulgaris* L., Fabaceae) to investigate whether *T. evansi* is able to manipulate JA and SA defences in this plant. In addition, whether it is true that *T. evansi* can suppress bean plant defences, suppression may have a positive effect on herbivore performance. To investigate this effect we evaluated the reproductive performance of *T. urticae* on bean leaves co-infested with *T. evansi*.

Hence, here we (i) investigated the manipulation of plant defences by *T. evansi* by measuring the phytohormone accumulation and expression levels of JA and SA defence-related genes in common bean leaves infested, co-infested and uninfested by *T. evansi* and *T. urticae* and (ii) investigated the effect of this manipulation in the reproductive performance of an herbivore by measuring the reproductive performance of *T. urticae* on leaves co-infested with *T. evansi*.

Material and Methods

Plants

The seeds of common beans (*Phaseolus vulgaris* L.) were sown in plastic pots (300mL) that contained soil substrate and were grown in a greenhouse with day/night temperatures of 18-23°C and a 16:8 (light: dark) regime until the plants were 10 days old. All plants were then transferred to a climate room at 25°C, 16:8h (light: dark) photoperiod and 60% relative humidity, where the experiments were carried out two days later. Hence, all the bean plants used in the experiments were 12 days old.

Spider mites

The strain of *T. evansi* used here is maintained on tomato leaves at the University of Amsterdam since 2010 and it was originally collected in 2002 from a population on tomato plants in a greenhouse at the Federal University of Viçosa (Sarmento *et al.* 2011a). *T. evansi* were transferred from tomato to bean leaves one month before the experiments. The *T. urticae* strain was obtained from a natural population collected from European spindle trees (*Euonymus europaeus*) in Santpoort (Noord-Holland, The Netherlands).

The two phytophagous mites were reared on detached bean leaves placed on cotton wool saturated with water inside plastic trays in a climate room at 25°C, 16:8 h (light:dark) and 60% relative humidity. Fresh leaves were provided 3 times per week. For experiments, adult female mites were taken from these rearing units and allowed to lay eggs on detached uninfested bean leaves on wet cotton wool to produce a cohort of eggs of the same age. After 48 hours, the adults were removed and the eggs were maintained under the same conditions as the colonies. Sixteen days later, 2-day-old adult females were collected from the cohorts and carefully transferred to the plants used in the experiments.

Phytohormone accumulation and gene expression upon spider mite attack

To investigate the extent to which *T. evansi* suppresses bean defences, we measured the phytohormone accumulation and gene expression of defence related genes on infested, co-infested and non-infested bean leaves. To evaluate the induction of plant defences by each spider mite species, one of the two leaves of a bean plant was infested with 30 *T. evansi* (TE) or 30 *T. urticae* (TU) females. To measure whether *T. evansi* would be able to suppress the plant defence otherwise induced by *T. urticae*, both spider mite species (TE + TU) were placed on one of the two leaves of a bean plant and allowed to freely walk and feed.

To investigate whether the suppression of plant defences by *T. evansi* is restricted to its feeding site, we divided bean leaves into two sections with a lanolin barrier and placed *T. evansi* on the bottom section of the leaf, which is the part of the leaf attached to the petiole (TE + TU (TE)) and *T. urticae* on the tip section (TE + TU (TU)). Control bean plants were kept uninfested (control). The mites placed on the leaves were allowed to feed for 48h and subsequently, the leaves were detached from the plants and immediately frozen in liquid nitrogen and stored at -80°C.

These bean leaves were checked for the concentrations of the main phytohormones involved in the jasmonate and salicylate pathways (see Fig. 1 and 2) and target genes regulated by both pathways. Thus, we measured the accumulation of the phytohormones TA, OPDA, JA, JA-Ileu and SA and the gene expression of JA and SA regulated genes. For the treatments (TE + TU (TE) and TE + TU (TU)) where mites were separated by a lanolin barrier, the bottom and tip parts of the leaf were cut with scissors and both leaf parts were frozen separately without the lanolin.

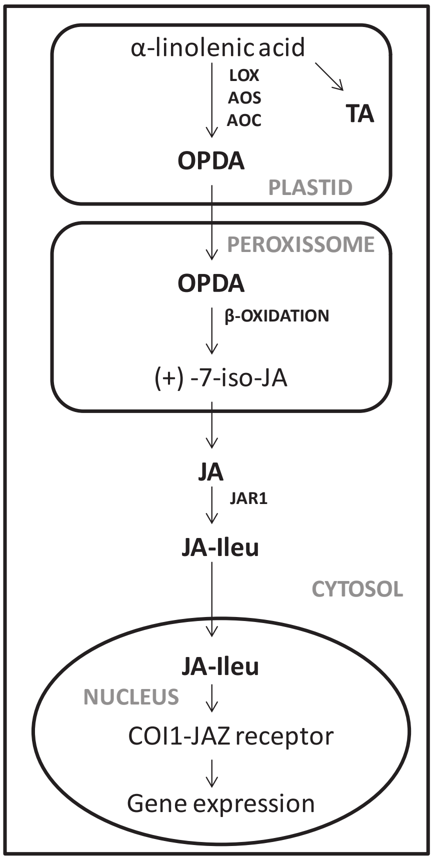

Figure 1 – Simplified schematic representation of the jasmonate pathway in plants. Based mainly on Croft *et al.* (1993), Wasternack *et al.* (2006) and Koo & Howe (2012). Biosynthesis of jasmonates starts in the plastids and involves the conversion of α-linolenic acid to 12-oxo-phytodienoic acid (OPDA) via the consecutive action of 13-lipoxygenase (LOX), 13-allene oxide synthase (AOS) and allene oxide cyclase (AOC), respectively. Alternatively, trans-10-ODA acid is synthesized from α-linolenic acid and through auto-oxidation forms traumatic acid (TA) (Zimmerman & Coudron, 1979), which is associated with wounding in plants. OPDA is then transported to the peroxissome and subjected to three cycles of β-oxidation to yield the 3R,7S stereoisomer of JA (also known as (+)-7-*iso*-JA). JA is transported to cytosol where it is conjugated specifically with the amino acid Ileu by the enzyme JAR1 and it gives rise to JA-Ileu. JA-Ileu synthesised in the cytosol presumably diffuses to the nucleus where it binds COI1-JAZ repressor complexes to activate the expression of JA-regulated genes.

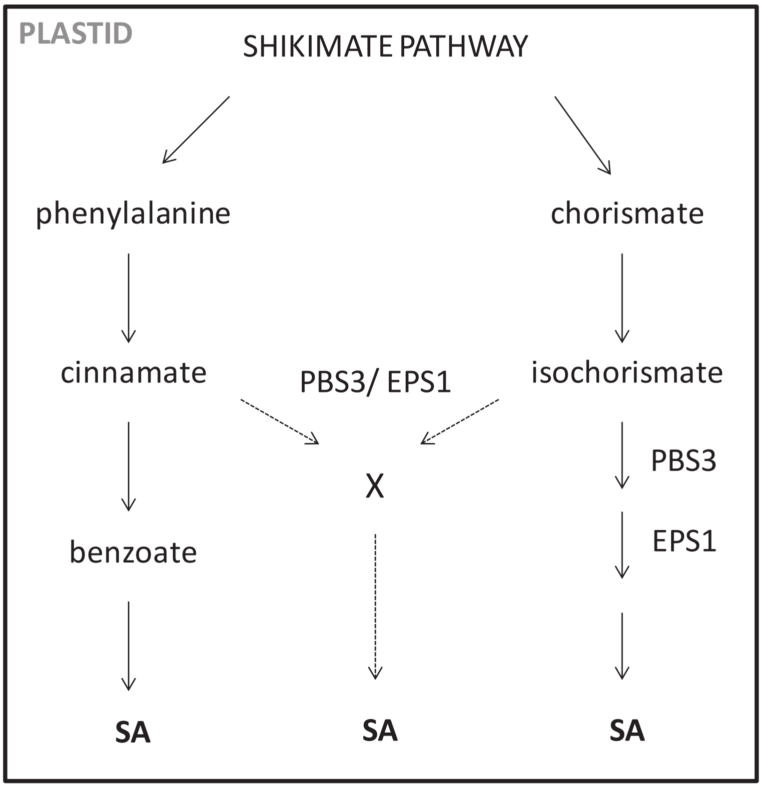

Figure 2 – Simplified schematic representation of the biosynthesis of salicylic acid (SA) in plants. Based mainly on Chen *et al.* (2009). It has been suggested that one way is to synthesize SA from cinnamate regulated by the phenylalanine, which is an end product of the shikimate pathway. The phenylalanine synthesizes SA with benzoate as an immediate precursor. Another way is through the shikimate pathway, with isochorismate as a precursor for the biosynthesis of SA. Due to the importance of both phenylalanine and isochorismate for SA biosynthesis a third way has been proposed and relies on unknown intermediates (X) as precursors from both the phenylalanine and isochorismate pathways to the SA biosynthesis. The recently identified PBS3 and EPS1 are important for pathogen-induced SA production and may encode enzymes catalyzing related, and possibly sequential, reactions in the synthesis of an important precursor or regulatory molecule for SA biosynthesis.

Phytohormone analysis

The bean leaves were treated as detailed above. Around 200 mg of frozen leaf material of each sample was ground in 1ml of ethyl acetate spiked with JA-D5 and SA-D6 as internal standards at a final concentration of 100 ng ml-1. Plant material was removed through centrifugation at 13000 rpm during 20 minutes. Supernatants were transferred to a new tube. To increase the recovery rate, the remaining plant material was cleaned with ethyl acetate without internal standards. The ethyl acetate fractions were pooled and evaporated in a concentrator (Labconco) at room temperature. It was used 500 µl of methanol 70% to re-dissolve phytohormones and internal standards.

Samples were analyzed with Liquid Chromatography (Varian ProStar) using a column chromatography Pursuit 5 C18 50x2.0 mm (Varian) coupled to a double Mass Spectrometer in tandem (Varian 320 MS-MS, http://varianic.com). Serial dilutions of pure compounds of JA, JA-Ileu and SA were used to estimate hormone concentrations and retention times. JA-D5 and SA-D6 values were used to calculate the recovery rate and correct the hormone concentration. The hormone concentration was estimated through integration of peak areas, corrected for the fresh weight of leaflet tissue. Hence, the results are given in ng per gram of fresh weight (ng/g FW).

Gene expression

We measured the relative expression of Wound-inducible Carboxypeptidase (*WICP*, a JA-regulated gene), Pathogenesis-related Protein 2 (*PRP-2*, SA-regulated) and Phenylalanine Ammonia-lyase 1 (*PAL-1*, SA-regulated) in bean plants treated as detailed above. Total RNA of a small part of the bean leaf was isolated by hot phenol (Invitrogen, http://www.invitrogen.com).

Integrity and purity of the nucleic acid was checked by NanoDrop (ND-1000; Thermo Scientific) analysis and agarose-gel, respectively. Four μg of total nucleic acid were treated with a Turbo-DNAse kit following the protocol recommended by the producer (Ambion, Turbo DNA-free kit http://www.ambion.com) to remove DNA. Two μg of DNA free samples were used for cDNA synthesis through M-MuLV reverse transcriptase, according to the manufacturer's instruction (Ferramentas Int. Burlington, Canada, RevertAid kit EP0441).

cDNA was diluted 10 times before use as template in real-time PCR. PCRs were performed in an ABI 7500 Real-Time PCR system (Applied Biosystems, http://www.appliedbiosystems.com), using the Platinum SYBR Green qPCR SuperMix-UDG kit (Invitrogen). Reactions were set in a volume of 20 μl, containing 0.25 μM of each primer, 0.1 μl ROX reference dye and 1 μl of cDNA (100 ng/μl). The cycling program was set to 5 min of pre-cycling stage (50°C), 5 min at 95°C, 45 cycles of 15 sec at 95°C and 1 min at 60°C, followed by a melting curve analysis.

For expression of *WICP* (ESTs annotation: CL794Contig1), which shows the highest similarity to a tomato *WICP* (GenBank: AF242849.1) we used the pair of primers F: AAACGCTTTTTGGCTGGAAT and R: TCTGACAGCAAACATCCAGAA, for *PAL-1* (GenBank: M11939.1) the pair of primers F: GACACACAAGTTGAAGCACCA and R: TGCAGCTTCTTAGCATCCTTC and for *PRP-2* (GenBank: X61364.1) the pair of primers F: GAAGATGAGCtCAAAGCTGGTAA and R: CAAGGATTGGCCAAAAGGTA. *Actin-11* (GenBank: CV529679.1) was used as reference gene (F: TGCATACGTTGGTGATGAGG and R: AGCCTTGGGGTTAAGAGGAG). Gene expression of the *Actin-11* was used to normalize and correct for variance in quality of RNA and quantity of input cDNA. Hence, the normalized expression (NE) was estimated by the ΔCt method [NE = $2^{-(\text{Ct target}-\text{Ct reference})}$]. To plot the relative expression, NE values were scaled by the group with lower NE.

Reproductive performance of *T. urticae* on leaves infested with *T. evansi*

To test if *T. evansi* suppression has a positive effect on herbivores performance, the reproductive performance of *T. urticae* in bean leaves infested with *T. evansi* was evaluated. To do so, we gently transferred fifteen adult female spider mites (2 days old) of either *T. urticae* or *T. evansi* to the bottom part of a bean leaf and five *T. urticae* to the tip part using a paintbrush.

A lanolin barrier was applied in the middle of the leaf to separate both group of mites. For control treatments, the bottom part of the leaf was left uninfested (no mites). Hence, the three treatments were: *T. urticae* x *T. urticae*, *T. evansi* x *T. urticae* and no mites x *T. urticae*. The spider mites were allowed to feed and reproduce and after 48h the females were removed from the tip of the leaves and the number of eggs laid was assessed using a stereomicroscope. Therefore, only the oviposition of *T. urticae* on the tip was assessed.

To estimate the reproductive performance of *T. urticae*, its oviposition rate was calculated taking into account the average number of eggs laid per female and per day. It was assessed by first dividing the total number of eggs found on each leaf by the number of females still present on these leaves at the end of the experiment. The number of eggs laid per female was then divided by the number of hours that the females were allowed to feed on each leaf and to calculate the number of eggs laid per day, the number of eggs laid per hour was multiplied by 24.

Statistical analysis

Phytohormone accumulation and gene expression upon spider mite attack - Before analysing the data of the accumulation of the phytohormones, we used Dixon's Q test to identify and exclude outliers in the phytohormone data. Latter, the accumulation of the phytohormones TA, OPDA, JA, JA-Ileu and SA was log-transformed and the effect of the treatments on the phytohormone accumulation was analysed with MANOVA. The treatments were included in the model as explanatory variable (x) and all the phytohormones were included in the model as response variables (y). Differences in the accumulation of each phytohormone individually among the treatments were analysed using generalized linear models (GLM) with a normal error distribution, with treatment as explanatory variable and the log-transformed phytohormone accumulation as response variable. For each treatment were carried out 2 to 5 replicates.

Gene expression - For the analysis of the expression of the target genes, the NE of the genes *WICP*, *PRP-2* and *PAL-1* were square root-transformed and the effect of the treatments on gene expression was analysed with MANOVA. The treatments were included in the model as explanatory variable and all the genes were included in the model as response variables. Differences in the square root-transformed NE of each target gene were compared independently using GLM with a normal error distribution. The NE of the defence genes was included as response variable and the treatments as explanatory variable. For each treatment were carried out 2 to 5 replicates.

Reproductive performance of T. urticae on leaves infested with T. evansi - The oviposition rate of *T. urticae* was Square root transformed, analysed using GLM with a Normal error distribution and included in the model as response variable.

The three treatments were included in the model as explanatory variable and were carried out 20 to 23 replicates per treatment.

Models were built by including all the variables and were checked with residual analyses to correct for overdispersion and distribution. Contrasts among treatments were calculated by aggregating non-significant ($p>0.05$) treatment levels in an *a posteriori* stepwise procedure (Crawley, 2007). All statistical analyses were done in R statistical software, version 2.15.1 (R Development Core Team, 2010).

Results

Phytohormone accumulation and gene expression vary upon spider mite attack

The accumulation of the phytohormones varied significantly on bean leaves infested, co-infested or non-infested by spider mites (MANOVA: Pillai-Bartlett statistic (Pillai) = 2.3; *df*= 5,25; P< 0.001, Fig. 1 and 2). The accumulation of the phytohormones TA, OPDA and SA was significantly affected by the treatment (ANOVA: TA: $F_{[5,16]}$= 3.1, P= 0.03, Fig. 1B; OPDA: $F_{[5,16]}$= 8.2, P< 0.001, Fig. 1C; SA: $F_{[5,16]}$= 88.1, P< 0.001, Fig. 2B), while JA was slight significantly affected (ANOVA: $F_{[5,16]}$= 2.6, P= 0.06, Fig. 1D) and JA-Ileu was not affected (ANOVA: JA-Ileu: $F_{[5,16]}$= 1.8, P= 0.17, Fig. 1E).

The accumulation of the phytohormones TA and OPDA among the treatments followed similar trends, which were not induced by TU infestation (GLM: TA: $F_{[1,19]}$= 0.1, P= 0.79; OPDA: $F_{[1,19]}$= 1.0, P= 0.33), but were strongly suppressed by TE infestation (GLM: TA: $F_{[1,20]}$= 7.5, P= 0.01; OPDA: $F_{[1,20]}$= 17.4; P< 0.001). However, accumulation on leaves co-infested with TE + TU, TE + TU (TE) and TE

+ TU (TU) did not differed from each other (GLM: TA: $F_{[1,20]}$= 0.6, P= 0.46; OPDA: $F_{[1,20]}$= 0.2; P= 0.69) but induced the accumulation of this hormone differently from control plants and plants infested with TU (GLM: TA: $F_{[1,20]}$= 5.3; P= 0.03; OPDA: $F_{[1,20]}$= 14.7; P< 0.001).

The phytohormone JA was induced by all the spider mite treatments, i. e. the control treatment was significantly different from all the other treatments (GLM: $F_{[1,21]}$= 11.0; P= 0.003). SA accumulation in bean leaves co-infested with TE + TU and TE + TU (TU) was similar to the accumulation upon TU infestation (GLM: $F_{[1,18]}$= 0.7; P= 0.42). When compared with Control, these treatments were highly induced (GLM: $F_{[1,19]}$= 332.0; P< 0.001), while infestation by TE induced SA accumulation in a lower degree (GLM: $F_{[1,19]}$= 9.0; P= 0.007).

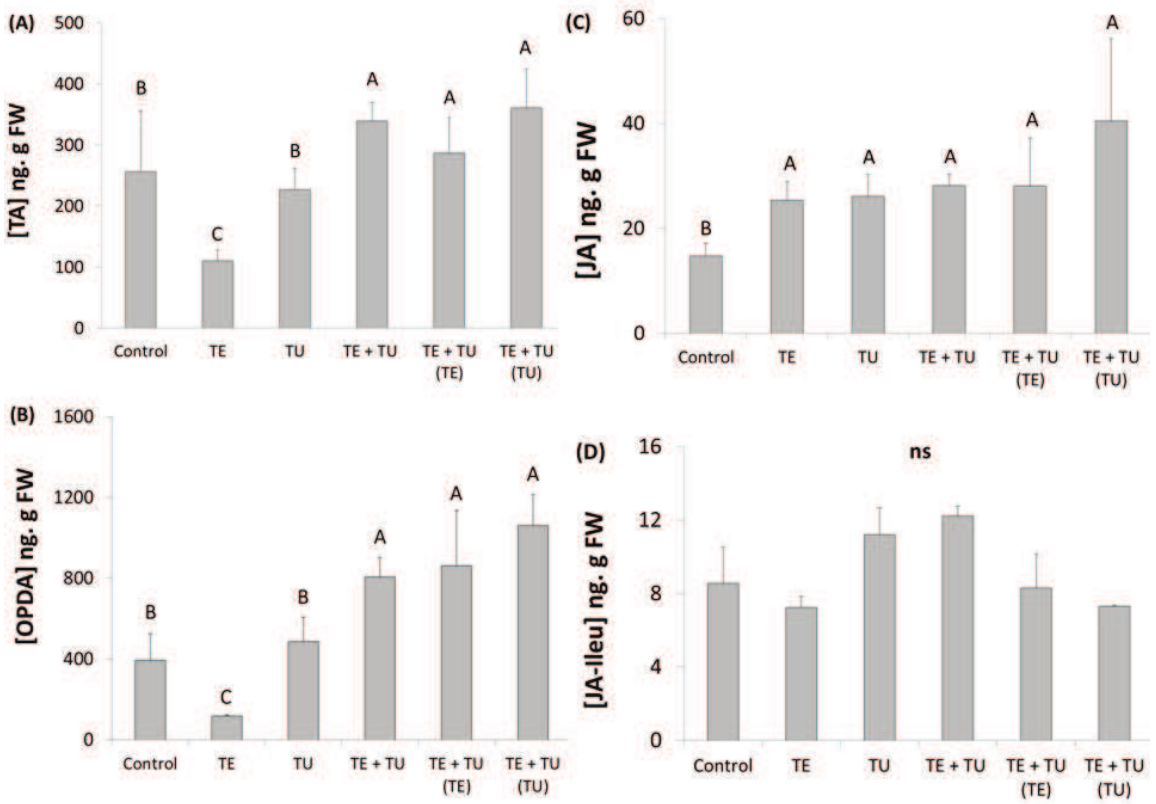

Figure 3 – Accumulation (±SEM) of the metabolites involved in the jasmonate pathway (see Fig. 1). (A) TA, **(B)** OPDA, **(C)** JA and **(D)** JA-Ile accumulation in bean leaves that were kept uninfested (Control), infested with spider mites (TE: infestation by 30 *T. evansi*; TU: infestation by 30 T. *urticae*) or co-infested with spider mites (TE+TU: 30 *T. urticae* + 30 *T. evansi*; TE+TU (TE): 30 *T. urticae* + 30 *T. evansi* separated by a lanolin barrier, bottom part of a bean leaf infested with *T. evansi* was sampled; TE+TU (TU): 30 *T. urticae* + 30 *T. evansi* separated by a lanolin barrier, tip part of a bean leaf infested with *T. urticae* was sampled). The bean leaves were sampled after 48h. Bars with different letters within each treatment differ significantly (P≤ 0.05); ns indicates non significant differences among the treatments and leaflets (P> 0.05).

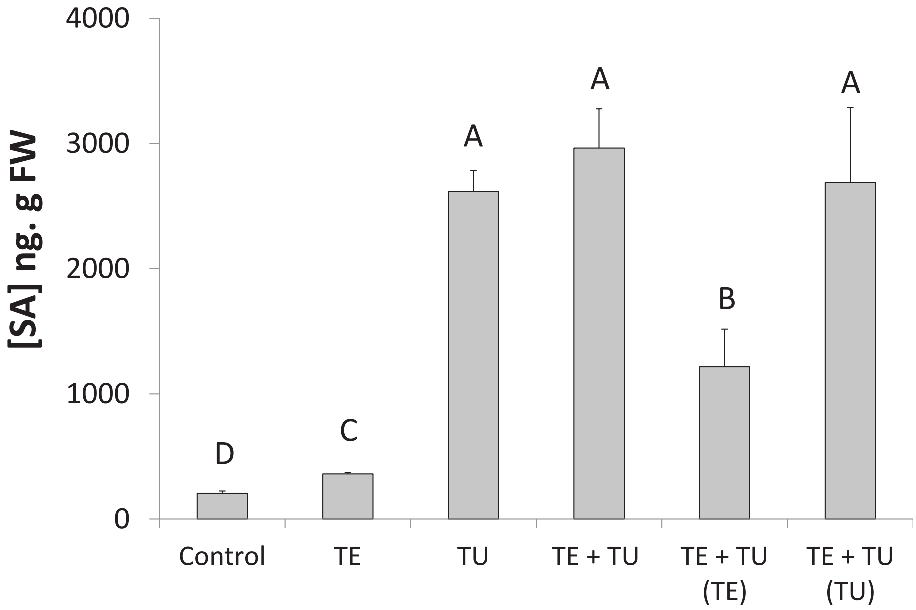

Figure 4 – Accumulation (±SEM) of the phytohormone SA involved in the salicylate pathway. Bean leaves were kept uninfested (Control), infested with spider mites (TE: infestation by 30 *T. evansi*; TU: infestation by 30 T. *urticae*) or co-infested with spider mites (TE+TU: 30 *T. urticae* + 30 *T. evansi*; TE+TU (TE): 30 *T. urticae* + 30 *T. evansi* separated by a lanolin barrier, bottom part of a bean leaf infested with *T. evansi* was sampled; TE+TU (TU): 30 *T. urticae* + 30 *T. evansi* separated by a lanolin barrier, tip part of a bean leaf infested with *T. urticae* was sampled). The bean leaves were sampled after 48h. Bars with different letters within each treatment differ significantly (P≤ 0.05); ns indicates non significant differences among the treatments and leaflets (P> 0.05).

The effect of the treatment on the expression of the genes *WICP, PRP-2* and *PAL-1* was significant (MANOVA: Pillai = 2.0; *df*= 5,15; P< 0.001, Fig. 3) as was the effect on the genes *PRP-2* and *PAL-1* separately (ANOVA: *PRP-2*: $F_{[5,15]}$= 18.2, P< 0.001; *PAL-1*: $F_{[5,15]}$= 18.2, P< 0.001). However, there was no effect of the treatment on the expression of the gene *WICP* (ANOVA: $F_{[5,15]}$= 2.2, P= 0.10).

The expression of the genes *PRP-2* and *PAL-1* followed similar trends. Both genes were not induced under infestation by TE (GLM: *PRP-2*: $F_{[1,17]}$= 0.0001, P= 0.98; *PAL-1*: $F_{[1,15]}$= 0.001; P= 0.97) neither by TE + TU (TE) (GLM: *PRP-2*: $F_{[1,18]}$= 0.5, P= 0.48; *PAL-1*: $F_{[1,16]}$= 3.1; P= 0.09). In contrary, both genes were highly induced after infestation by TU (GLM: *PRP-2*: $F_{[1,19]}$= 35.9, P< 0.001; *PAL-1*: $F_{[1,17]}$= 57.8, P< 0.001). The expression of these two genes in bean leaves co-infested with TE + TU was slight lower, but not statistically different from bean leaves under TU infestation (GLM: *PRP-2*: $F_{[1,19]}$= 0.4, P= 0.53; *PAL-1*: $F_{[1,17]}$= 2.1; P= 0.16).

Considering that *PAL* is involved in SA accumulation (Mauch-Mani & Slusarenko, 1996) here we correlated the activity of *PAL-1* gene with SA accumulation (Fig. 4) to evaluate how both spider mite species cope with SA defences in bean plants. Our results show that the SA accumulation and the expression of the gene *PAL-1* (SA-regulated) were similarly induced among the treatments, following similar trends. Thus, SA defence was highly induced by *T. urticae* (TU, Fig. 4) and it was not induced or at least induced in lower levels by *T. evansi* (TE, Fig. 4). However, the accumulation of SA in leaves infested with both species (TE + TU, light gray bars; Fig. 4) was similar (and even a slight higher) than leaves upon *T. urticae* infestation (TU, light gray bars; Fig. 4). In contrast, the expression of the gene *PAL-1* in leaves infested with both species (TE + TU, dark gray bars; Fig. 4) was induced the in lower levels than leaves infested only by *T. urticae* (TU, dark gray bars; Fig. 4).

The gene *WICP* is known to be activated in response to wounding, systemin and methyl jasmonate in a pattern similar to that shown for PIs genes (Moura *et al.*, 2001) and JA-Ileu is considered the main active biological compound of the JA pathway. Therefore, here we correlated *WICP* expression with JA-Ileu accumulation (Fig. 5) to evaluate the how both spider mite species cope with JA defences in bean plants. Besides the fact that gene expression or phytohormone accumulation was not significantly different induced among the treatments, the correlation between JA-Ileu and *WICP* could not be as clearly observed as the correlation between *SA* and *PAL-1*.

Reproductive performance of *T. urticae* increases on leaves infested with *T. evansi*

The reproductive performance of *T. urticae* was differentially affected among the treatments (GLM, $F_{[2,63]}$=17.4; P< 0.001; Fig. 6). *T. urticae* reproductive performance was higher when sharing a leaf with *T. evansi* than when sharing a leaf with *T. urticae* (GLM, $F_{[1,63]}$= 34.4; P< 0.001) or on uninfested leaves (GLM, $F_{[1,63]}$= 13.0; P< 0.001).

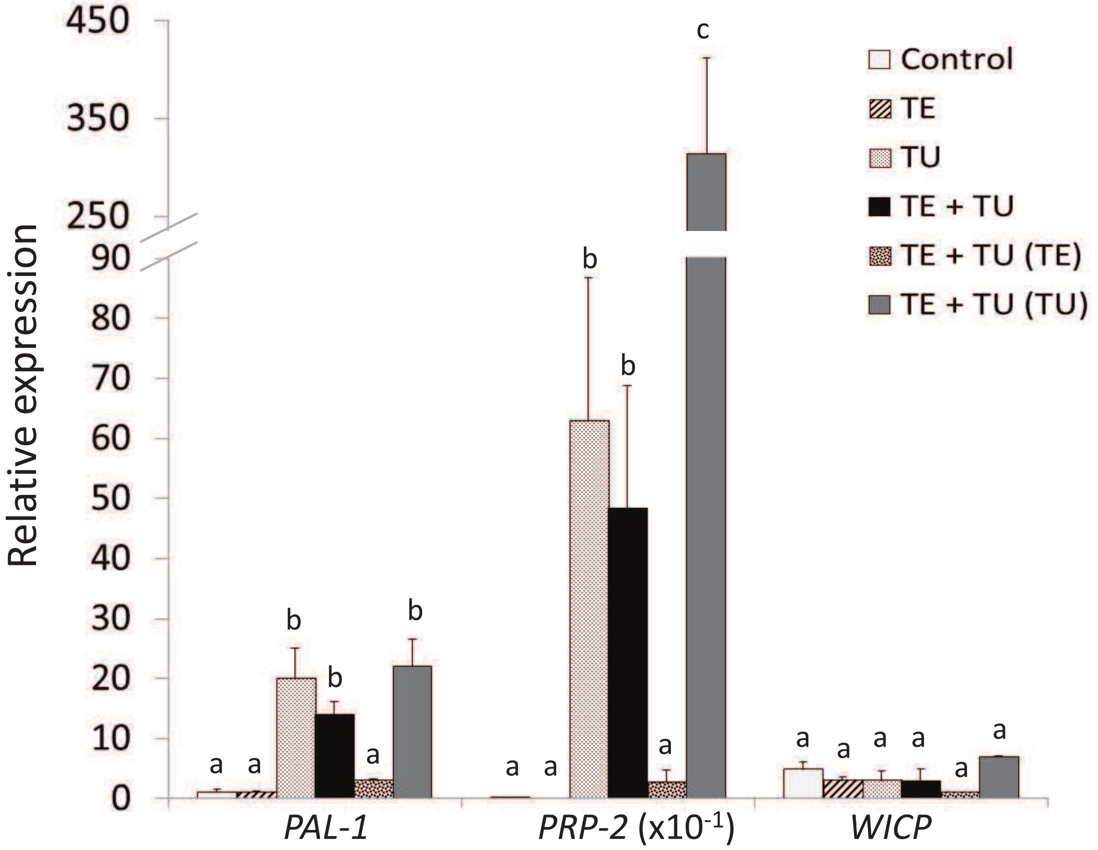

Figure 5 – Expression of genes regulated by jasmonic acid (JA) and salicylic acid (SA) pathway in non-infested or infested bean leaves. Relative expression (±SEM) of the genes *WICP* (JA-regulated), *PRP-2* and *PAL-1* (SA-regulated) in uninfested bean leaves (Control), bean leaves infested with spider mites (TE: infestation by 30 *T. evansi*; TU: infestation by 30 T. *urticae*) and co-infested with spider mites (TE+TU: 30 *T. urticae* + 30 *T. evansi*; TE+TU (TE): 30 *T. urticae* + 30 *T. evansi* separated by a lanolin barrier, bottom part of a bean leaf infested with *T. evansi*; TE+TU (TU): 30 *T. urticae* + 30 *T. evansi* separated by a lanolin barrier, tip part of a bean leaf infested with *T. urticae*). Bars with different letters within each gene differ significantly (P≤ 0.05).

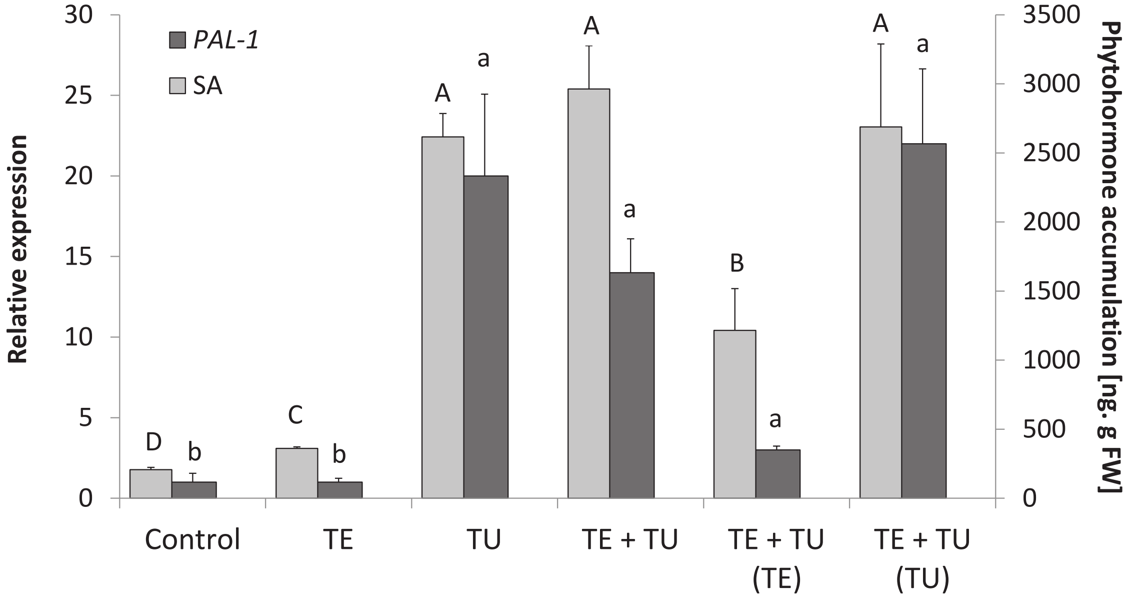

Figure 6 – Link among phytohormone accumulation and gene expression. Accumulation (±SEM) of the phytohormone SA (light grey bars) and the relative expression (±SEM) of *PAL-1* (dark grey bars) in non-infested or infested bean leaves. Bean leaves were kept uninfested (Control), infested with spider mites (TE: infestation by 30 *T. evansi*; TU: infestation by 30 T. *urticae*) or co-infested with spider mites (TE+TU: 30 *T. urticae* + 30 *T. evansi*; TE+TU (TE): 30 *T. urticae* + 30 *T. evansi* separated by a lanolin barrier, bottom part of a bean leaf infested with *T. evansi* was sampled; TE+TU (TU): 30 *T. urticae* + 30 *T. evansi* separated by a lanolin barrier, tip part of a bean leaf infested with *T. urticae* was sampled). Bars with different letters (uppercase phytohormone, lowercase gene expression) differ significantly (P≤ 0.05) among treatments.

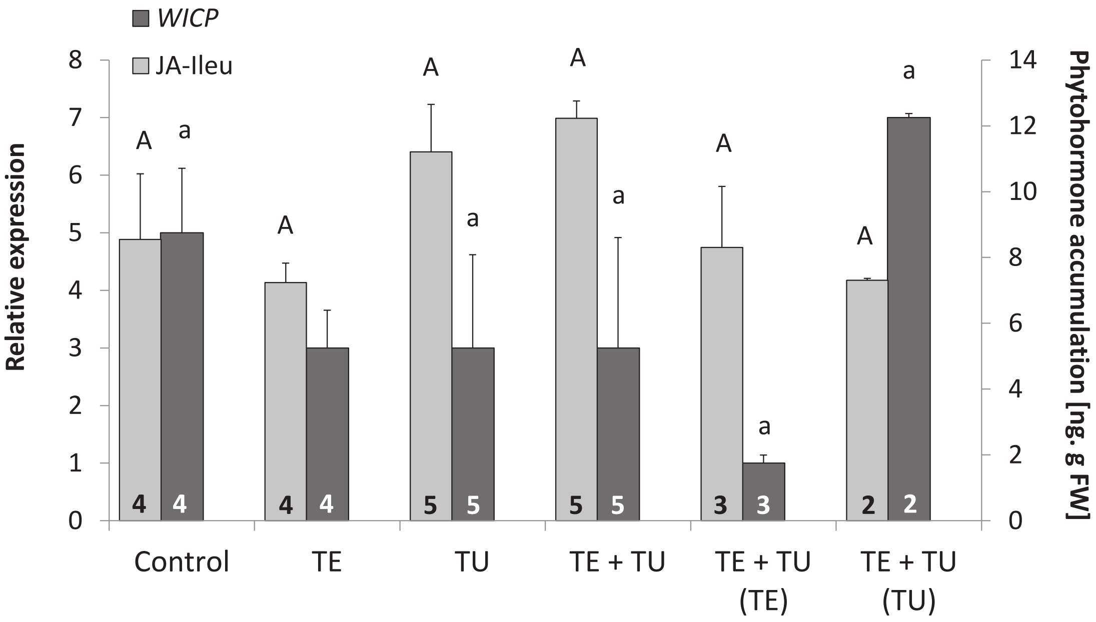

Figure 7 – Link among phytohormone accumulation and gene expression. Accumulation (±SEM) of the phytohormone JA-Ileu (light grey bars) and the relative expression (±SEM) of *WICP* (dark grey bars) in non-infested or infested bean leaves. Bean leaves were kept uninfested (Control), infested with spider mites (TE: infestation by 30 *T. evansi*; TU: infestation by 30 T. *urticae*) or co-infested with spider mites (TE+TU: 30 *T. urticae* + 30 *T. evansi*; TE+TU (TE): 30 *T. urticae* + 30 *T. evansi* separated by a lanolin barrier, bottom part of a bean leaf infested with *T. evansi* was sampled; TE+TU (TU): 30 *T. urticae* + 30 *T. evansi* separated by a lanolin barrier, tip part of a bean leaf infested with *T. urticae* was sampled). Bars with different letters (uppercase phytohormone, lowercase gene expression) differ significantly (P≤ 0.05) among treatments.

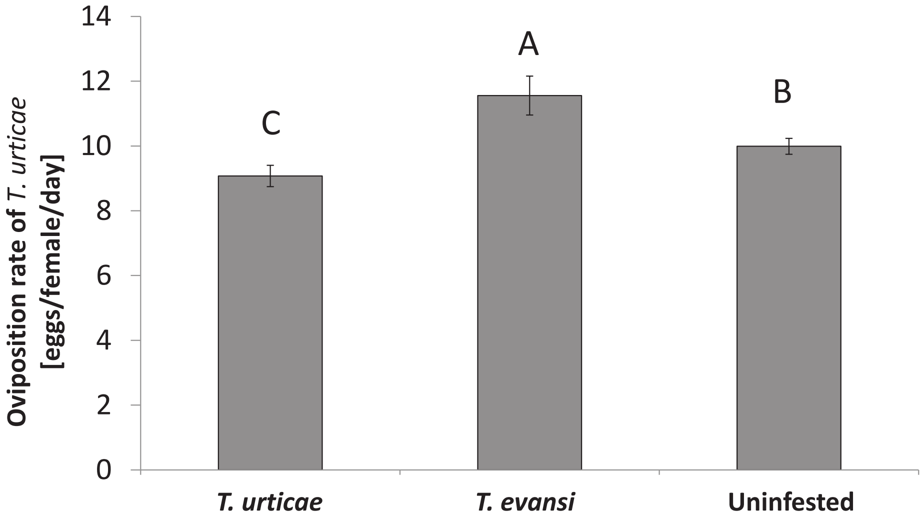

Figure 8 – Oviposition performance of *T. urticae*. The bottom part a bean leaf was infested with fifteen *T. evansi* or with fifteen *T. urticae* or left uninfested and at the tip part of the leaf, three *T. urticae* female were allowed to feed and produce eggs. After 48h the number of eggs laid by *T. urticae* at the tip was assessed. *T. urticae* oviposition rate (±SEM) was higher when sharing a leaf with *T. evansi* than when sharing a leaf with *T. urticae* or on uninfested leaves. Different letters denote significant differences ($P \leq 0.05$) among treatments.

Discussion

In one hand, our results demonstrate that *T. evansi* was able to manipulate the SA plant defences, but in the other hand, the manipulation of JA defences only occurred in the early steps of the JA pathway and hence was not fully elucidated. In addition, we showed that the reproductive performance of *T. urticae* increased in the presence of *T. evansi*, suggesting that suppression of bean plant defences by *T. evansi* occurred and that it consequently had a positive effect on the performance of the spider mite *T. urticae*.

In *Phaseolus vulgaris*, the expression pattern of *PAL* genes in response to pathogen infection or elicitor treatment has been documented (Lawton & Lamb, 1987; Jakobek & Lindgren, 1993). Here, we demonstrated the expression pattern of a *PAL* gene (*PAL-1*) in bean leaves under spider mites attack and due to its role in SA synthesis (Mauch-Mani & Slusarenko, 1996) we correlated *PAL-1* activity with SA accumulation (Fig. 4). Our results showed that SA defence was highly induced by *T. urticae* (TU, Fig. 4) and it was not induced or at least induced in lower levels by *T. evansi* (TE, Fig. 4).

Since leaves co-infested by *T. evansi* and *T. urticae* (TE + TU, light gray bars; Fig. 4) induced the accumulation of SA in similar levels found in plants upon infested by *T. urticae* (TU, light gray bars; Fig. 4), it indicates that *T. evansi* did not suppress the SA accumulation induced by *T. urticae.* Therefore, non-induction instead of active suppression of SA accumulation would better explain why the concentration of this phytohormone upon co-infestation (TE + TU, light gray bars; Fig. 4) is similar to the one found after infestation with *T. urticae* (TU, light gray bars; Fig. 4) instead of being significantly lower as would have been expected with suppression. Hence, feeding by *T. evansi* does not cause an increase in SA accumulation or causes only a slight increase.

In contrast, leaves infested by *T. urticae* and *T. evansi* together (TE + TU, dark gray bars; Fig. 4) induced the expression of the gene *PAL-1* in low levels than leaves infested only by *T. urticae* (TU, dark gray bars; Fig. 4), indicating that in this case *T. evansi* partially suppressed the expression of *PAL-1* induced by *T. urticae*. Therefore, these results suggest that *T. evansi* mediated SA defence suppression in beans, as well as found previously on tomato (Alba *et al.*, in preparation), and this operates at the level of gene expression.

It has been reported that an increase in SA accumulation coincides with the expression of a large number of PRs proteins (Ward *et al.*, 1991). Similarly, here we report that the expression of the gene *PRP-2* was well correlated with SA accumulation. Considering that the genes *PAL-1* and *PRP-2* follow identical expression patterns, these results reinforce the assumption that suppression by *T. evansi* occurs downstream phytohormone biosynthesis. Furthermore, we can suggest that low SA accumulation and transcript levels of the SA-defence marker genes *PAL-1* and *PRP-2* are representative biomarkers of SA suppression by *T. evansi*. However, how *T. evansi* manipulates plant defences is unknown.

One possible explanation could be that when *T. evansi* feeds on a bean leaf it introduces some effectors that prevent the plant to trigger the activation of SA-related genes or the accumulation of SA and such effectors are probably absent in *T. urticae* saliva. Although the presence of effectors in *T. evansi* saliva would explain the low transcript levels of genes like *PAL-1* and *PRP-2*, other possible functions of effectors should be considered. For example, they might directly bind and inhibit defensive proteins and these targets might not be only restricted to plant defences.

Alba *et al.* (in preparation) have shown that other biological processes such as tomato leaf senesce is induced by *T. urticae* and suppressed by *T. evansi*. This indicates that suppression caused by *T. evansi* is not only affecting the defence response of the plant but also could interfere with other plant physiological process different from defences. Therefore, suppression could be a size effect of other processes that are interacting with plant defences.

Defence suppression does not necessarily mean that the expression of all plant genes is suppressed. As an example, Alba *et al.* (in preparation) showed that the *GAME1* (a gene involved in the glycosylation of steroidal alkaloids, thereby reducing the toxicity of alkaloids) was induced by *T. evansi*, whereas *T. urticae*-infested tomato plants did not. The up-regulation of the gene *GAME1* by *T. evansi* may reduce the potential toxic effect of alkaloids and consequently, a decrease in tomato alkaloid concentrations is expected to have a positive effect on the fitness of *T. evansi* and even on the fitness of other herbivores. Alba *et al.* (in preparation) even suggest that *T. evansi* might manipulate the plant's own regulatory system to enhance those processes that are beneficial and to repress the harmful ones.

The suppression of JA pathway by *T. evansi* was only observed in the early steps of the JA pathway, where the accumulation of phytohormones TA and OPDA were clearly suppressed (TE, Fig. 1B and C). However, the phytohormones JA and JA-Ileu were not significantly suppressed by *T. evansi* (TE, Fig. 1D and E). In addition, the JA-regulated gene *WICP* was also not significantly suppressed by *T. evansi* (TE, Fig. 5) and was expressed similarly in all the treatments (Fig. 5).

Since it has been shown that *T. evansi* is able to manipulate JA defences in tomato plants (Sarmento *et al.* 2011; Alba *et al.*, in preparation) the lack of effect found for JA and JA-Ileu accumulation and *WICP* expression on bean leaves under *T.*

evansi attack in the current study is surprisingly. One possible explanation would be that the JA pathway has not been activated in bean plants to overcome spider mite attack. However, it has been shown that JA accumulation increases in bean leaves after fungus inoculation (Cavallo & Raggi, 2002) and virus infection (Clarke *et al.*, 2000), which indicates the JA defence has been activated to increase plant defences against pathogens. In addition, peroxidase (PDO) and polyphenol (PPO) activity were found to be induced by exogenous JA application and by herbivory in tomato plants (Thaler *et al.*, 1996; Cipollini Jr & Redman, 1999) as well as in bean plants after the application of a herbivore oral secretion (Kruzmane *et al.*, 2002).

Summarizing, JA defences increases in bean leaves under pathogen attack and herbivore oral secretion and JA defences are activated under spider mites attack in tomatoes (Li *et al.*, 2002; Ament *et al.*, 2004; Sarmento *et al.*, 2011a). Therefore, we expected that bean leaves under spider mite attack would also activate JA defences, but this was not the case. Considering the possibility that the JA-marker that we chose to study the activation of JA defences in bean plants under spider mite feeding was not the most suitable, in a near future, experiments using different JA-defence marker genes should be carried out.

Our oviposition experiments (Fig. 6) showed that the oviposition performance of *T. urticae* is higher when it shares a leaf with *T. evansi* suggesting that as well as previously shown on tomato plants (Sarmento *et al.*, 2011a), suppression of plant defences by *T. evansi* occurs and promotes the reproductive performance of a herbivore.

Since the two spider mite species were physically separated by a lanolin barrier, these results suggest that suppressed defences are not restricted to *T. evansi* feeding site, but it also occurs in the adjacent part of the leaf and consequently the low levels of defensive compounds in *T. urticae* feeding site promotes an increase in its reproductive performance. However, defence suppression might consist also in disadvantages for *T. evansi*. For example, *T. evansi* manipulation of plant defences might have a cost and suppression of plant defences beyond its feeding site may facilitate competitors, which may profit from these investments without having invested in such traits.

Indeed, it has been shown that the closely related spider mite *T. urticae* profits from the increase in plant quality caused by *T. evansi* feeding on tomato (Sarmento *et al.*, 2011b) and here we show the same outcome on bean plants. Hence, one might think that suppression of plant defences by *T. evansi* can backfire, as it makes plants more profitable to competitors, like *T. urticae*. However, to evaluate the effects of plant suppression by *T. evansi*, other strategies employed by this herbivore have to be also considered.

Sarmento *et al.* (2011b) has shown that *T. evansi* is able to monopolize its feeding site by the production of massive quantities of web, which is hard to be penetrated by competing mites and it would serve to refrain the population growth of its competitors. Indeed, it was shown that when *T. evansi* and *T. urticae* were simultaneously introduced in tomato plants, the population of *T. urticae* went extinct. Therefore, investigations of whether herbivores profit from suppression of plant defences by *T. evansi* either in tomato, bean or other plants are still part of an open field.

There are abundant evidence for adaptation of insects to their food plants (Schoonhoven *et al.*, 2005) and since *T. evansi* is a specialist of Solanaceae (Migeon *et al.*, 2009) perhaps over the years *T. evansi* might have adapted to overcome host plant defences by down-regulating their defence system. Thus, in one hand it would not be a surprise if its ability to suppress plant defences is exclusive to its host plants. On the other hand, because JA and SA are widespread employed by the plant kingdom, if *T. evansi* is able to suppress these plant defences in tomato, it is likely that *T. evansi* suppresses JA and SA in bean plants as well.

Although here we did not clearly show that *T. evansi* manipulates JA defences in bean plants, due to the similarities of our results with previous reports of suppression using tomato plants, *T. evansi* ability to suppress plant defences seems to be a wide spread mechanism, which might be found on host plants, such as tomato and alternative host plants such as bean.

Acknowledgements

We thank CAPES, CNPQ and FAPEMIG for financial support.

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CHAPTER 3

Effects of jasmonate-induced tomato defences on herbivorous mites and their predators

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Abstract

Jasmonates (JA) play a key role in activating plant defences against arthropod herbivores. Herbivores like the two-spotted spider mite *Tetranychus urticae* induce JA-defences in tomato and these defences lower their reproductive performance. However, some herbivores like the red spider mite *Tetranychus evansi* have adapted to suppress plant defences to uphold a high reproductive performance. Here we used a defence-rescued protocol of the tomato JA-biosynthesis mutant *def-1* to first quantify the relationship between spider mite reproductive performance and jasmonate defences. Second, we tested to which extent these defences affected subsequently the feeding preference and performance of predatory mites preying on the eggs of these spider mites. First, our data showed that the reproductive performance of both spider mite species is negatively affected by JA-defences. Second, when we offered to the predatory mite *Phytoseiulus longipes* eggs from spider mites reared on plants with high or low JA-defences we observed that they ate more eggs from spider mites which had been feeding on plants with low JA levels. Moreover, predators preying on *T. urticae* eggs from plants with low JA-levels compared to plants with high levels also had a higher reproductive performance of the predator itself, while the predation on *T. evansi* eggs did not affect the reproductive performance of the predator. Subsequently, we offered predatory mites a choice between the eggs from *T. urticae* derived from the *def-1* mutant or from normal wild type tomatoes and the same we did with *T. evansi* eggs. While the predator preferred *T. urticae* eggs derived from *def-1* over those from wild type, it did not discriminate between the *T. evansi* eggs. Taken all together, we could show that both JA-inducing *T. urticae* and the JA-suppressing *T. evansi* mites are similarly sensitive to JA-defences and, hence, that defence suppression can be beneficial for spider mites. However, we also show that predatory mites eat more prey eggs when these came from JA-undefended plants when offered a choice between these and eggs from JA-defended plants. It suggests that defence suppression by *T. evansi* can backfire since it makes its eggs more palatable to predators. However, we argue that for evaluating the costs and benefits of defence suppression more research is needed.

KEYWORDS – plant resistance, plant susceptibility, plant defence, predation, tomato, *T. evansi*, *T. urticae*

Introduction

Plants are constantly pressured by herbivores to display defensive traits that maximize their own reproductive success (Karban & Myers, 1989). Two major strategies can be employed by the plant to reduce herbivore fitness: direct defences through the production of anti-herbivore compounds and indirect defences through the production of volatiles compounds that attract natural enemies of herbivores (Karban & Baldwin, 1997). Furthermore, each strategy can be constitutively displayed, i. e. are always present in a plant, or be induced, i. e. are activated only after attack and render plants more resistant to subsequent herbivory (Karban & Baldwin, 1997; Arimura *et al.*, 2005).

Central players to promote the induction of plant defence are a set of phytohormones that mediate herbivore recognition and establishment of defences. In particular, the plant hormones jasmonic acid (JA) and salicylic acid (SA) play primary roles in mediating these steps (Wu & Baldwin, 2010). These hormones can act individually, and have distinct effects in modulating inducible defences or interact with each other via cross-talk mechanisms (Koornneef & Pieterse, 2008). While JA is essential against chewing insects and cell content feeders (Walling, 2000), SA is mainly involved in defence against biotrophic pathogens (Robert-Seilaniantz *et al.*, 2011). However, defence against spider mites is characterized by simultaneous activation of both SA and JA pathways (Kant *et al.*, 2004).

Especially the JA-pathway has been characterized in detail and the JA-derivative jasmonoyl-isoleucine (JA-Ileu) appeared as the major bioactive form of JA and is related with the expression of typical defence genes (Li *et al.*, 2002; Koo & Howe, 2012), leading to the biosynthesis of defensive proteins such as proteinase inhibitors (PIs) (Green & Ryan, 1972) and polyphenol oxidases (PPOs) (Constabel *et al.*, 1995). Since PIs alter digestive processes, they interfere with growth and development of herbivores when present in high concentrations and play a potent defensive role against phytophagous insects (Ryan, 1990).

The JA-pathway is not only involved in direct plant defence, but it is also in indirect defences (Dicke *et al.*, 1999; Gols *et al.*, 2003), which can be simultaneous expressed by the plant and undergo tritrophic effects (Rasmann *et al.*, 2011).

Tritrophic interactions may include direct effects of host plants on herbivores and direct or indirect effects of host plants on natural enemies (Kahl *et al.*, 2000; Gassmann *et al.*, 2010).

While plants have evolved these sophisticated mechanisms, herbivores in turn have evolved counter-adaptations to overcome them to increase their performance (Karban & Agrawal, 2002). For instance, herbivores may have adapted to sequester the defensive chemicals of the host plant for their own protection (Karban & Baldwin, 1997) or dispose them via (enzymatic) degradation, modification or secretion (Schoonhoven *et al.*, 2005). Moreover, some herbivores and pathogens have adapted to manipulate plants to their own benefit, such that establishment of induced defences is reduced or prevented.

Kant *et al.* (2008) found a strain of *Tetranychus urticae* that did not induce JA-related defences in wild type tomato plants. This is surprising because plant feeding by most strains of this herbivore up-regulates the transcription of JA-related defence genes, and this response has been found responsible for reducing their reproductive performance on tomato plants (Li *et al.*, 2002; Ament *et al.*, 2004; Kant *et al.*, 2004).

Sarmento *et al.* (2011a) found that *T. urticae'* s sister species *Tetranychus evansi* performed much better on tomato leaves that were previously attacked by conspecifics. Likewise, a defence-inducing strain of *T. urticae* doubled its reproduction on leaves previously damaged by *T. evansi* (Sarmento *et al.*, 2011b). Since *T. evansi*-infested plants were found to have suppressed transcript levels and enzymatic activities of PIs (Sarmento *et al.*, 2011a), it is possible that JA signalling is prevented or suppressed by this mite. If so, the increased mite performance may be due to the plant failing to establish general JA defences.

Considering the role of JA in the establishment of direct and indirect plant defences, the JA signalling might undergo tritrophic effects and affect not only the herbivores but also the natural enemies of herbivores. For instance, Thaler (1999) demonstrated that JA plant defences affected parasitoid performance on tomato plants. In this study, parasitic wasps had reduced pupal weight and increased developmental time after feeding on caterpillars reared on induced tomato plants. This suggests that the parasitoid was indirectly affected by the plant defence through altered herbivore quality.

We argue here that JA plant defences may affect the spider mites *T. evansi* and *T. urticae* and subsequently, the predatory mite *Phytoseiulus longipes*. To investigate the function of JA in the mite-tomato interaction in more detail we used a mutant tomato plant (*def-1*) unable to induce JA accumulation and defensive proteins upon wounding or herbivory (Ament *et al.*, 2004). Due to the lack of ability to induce JA accumulation, *def-1* is considered as a susceptible genotype and spider mites are expected to have increased performance after feeding on this plant.

It has been shown that exogenous JA application can rescue the JA accumulation and plant resistance (Li *et al.*, 2002). Hence, we rescued the JA response in the *def-1* by supplying exogenous JA and Ileu to *def-1* leaflets and used them as resistant plants. So, we (i) quantified the effect of JA defences on the reproductive performance of the spider mites *T. evansi* and *T. urticae* and then (ii) tested how these defences affect the behaviour and performance of the predatory mite *P. longipes* preying on eggs produced by these herbivores. In doing so, we aimed to get a better insight in the costs and benefits of defence suppression within simple semi-natural communities.

Materials and Methods

Plants

The seeds of wild type (*Solanum lycopersicum* cv. Castlemart) and mutant *def-1* (*Solanum lycopersicum*) tomatoes were grown in a greenhouse with day/night temperatures of 18-23°C and a 16:8 (light:dark) regime. Plants (28 days old) were transplanted to plastic pots (2L) with soil. Two days prior to each experiment, all plants were transferred to a climate room at 25°C, 16:8h (light:dark) photoperiod and 60% relative humidity, where the experiments were carried out.

Mite rearings

The *T. urticae* strain used here was obtained from a natural population collected from European spindle trees (*Euonymus europaeus*) in Santpoort (Noord-Holland, The Netherlands). The strain of *T. evansi* used here is maintained at the University of Amsterdam since 2010. It was originally collected in 2002 from a population on tomato plants in a greenhouse at the Federal University of Viçosa (Sarmento *et al.*, 2011a).

The two phytophagous mites were reared on detached leaves placed on cotton wool saturated with water inside plastic trays in a climate room at 25°C, 16:8 h (light:dark) and 60% relative humidity. Fresh leaves were provided 3 times per week. *T. evansi* was reared on tomato leaves, *T. urticae* on bean leaves. For experiments, adult female mites of both species were taken from these rearing units and allowed to lay eggs on detached uninfested bean leaves on wet cotton wool to produce a cohort of eggs of the same age. After 48 hours, the adults were removed and the eggs were maintained under the same conditions as the colonies. Sixteen days later, 2-day-old adult females were collected from the cohorts and carefully transferred to the plants used in the experiments.

The colony of the predatory mite *P. longipes* was started in 2011 with individuals that were provided by the company Koppert Biological Systems, The Netherlands and it was maintained in a climate room at 25 °C, 16:8 h (light:dark) photoperiod and 60% relative humidity, supplied with detached cucumber leaves (*Cucumis sativa* L. var. Ventura) infested with *T. urticae* (a different line from the one used in the experiments). Although *P. longipes* is a predatory mite that can prey on *T. urticae*, in South Brazil *P. longipes* has been found to be associated with *T. evansi* (Furtado *et al.*, 2006), but not with *T. urticae*.

Cohorts of eggs of predators were obtained by placing adult females on detached cucumber leaves on wet cotton wool to produce a cohort of eggs of the same age. After 24 hours the females were removed and the eggs were maintained on the leaves under the same conditions as the colonies. The predatory females were used when 8 days old since egg.

Effect of the jasmonate defences on the reproductive performance of spider mites

We used *def-1* mutant tomato plants to investigate the effect of the JA defences on the reproductive performance of the spider mites *T. evansi* and *T. urticae.* To do so, we first detached five leaflets from the third leaf of *def-1* tomato plants (28 days old) and randomly submitted these leaflets to three treatments: (i) leaflets from *def-1* mutant plants that were placed in only tap water (the “water” treatment), (ii) leaflets placed in tap water + 1 mM L-Isoleucine (Ileu) (Sigma-Aldrich) (“JA-free”), and (iii) leaflets placed in tap water, 1 mM Ileu and 0.05 mM (±)-jasmonic acid (JA) (Sigma-Aldrich) (“JA-rescued”). In the last treatment, we mixed water, Ileu and JA to increase the levels of JA-Ileu in tomato leaflets (Orenes, 2011). Final solutions were prepared in tap water from a stock solution of JA dissolved in 70% methanol and a filter sterilized solution of Ileu dissolved in sterilized distillate water.

Orenes (2011) previously showed that there was a burst in the JA and JA-Ileu accumulation 24h after the immersion of leaflets in a solution with JA plus Ileu. After this time, the concentration of these compounds decreased to approximate half of the "burst" concentration and remained constant until they disappeared after 5 days. We therefore, using a paintbrush gently transferred 15 adult female spider mites of either *T. urticae* or *T. evansi* to each leaflet 24h after the leaflet treatment where they were allowed to feed and reproduce for 48h (see Fig. 1). Subsequently, the spider mite females were removed from the leaflets and the number of eggs laid was assessed using a stereomicroscope.

To prevent the mites from escaping, insect glue was applied around the leaflet petiole. All the spider mites used in this experiment were previously subjected to the same treatment for 48h (Agrawal *et al.*, 2002). To check the concentrations of the phytohormones JA-Ileu, JA and SA (see below) effectively taken up and to investigate their effect on spider mites reproductive performance, the leaflets were removed from the solutions, immediately frozen in liquid nitrogen and stored at -80°C until phytohormone analysis.

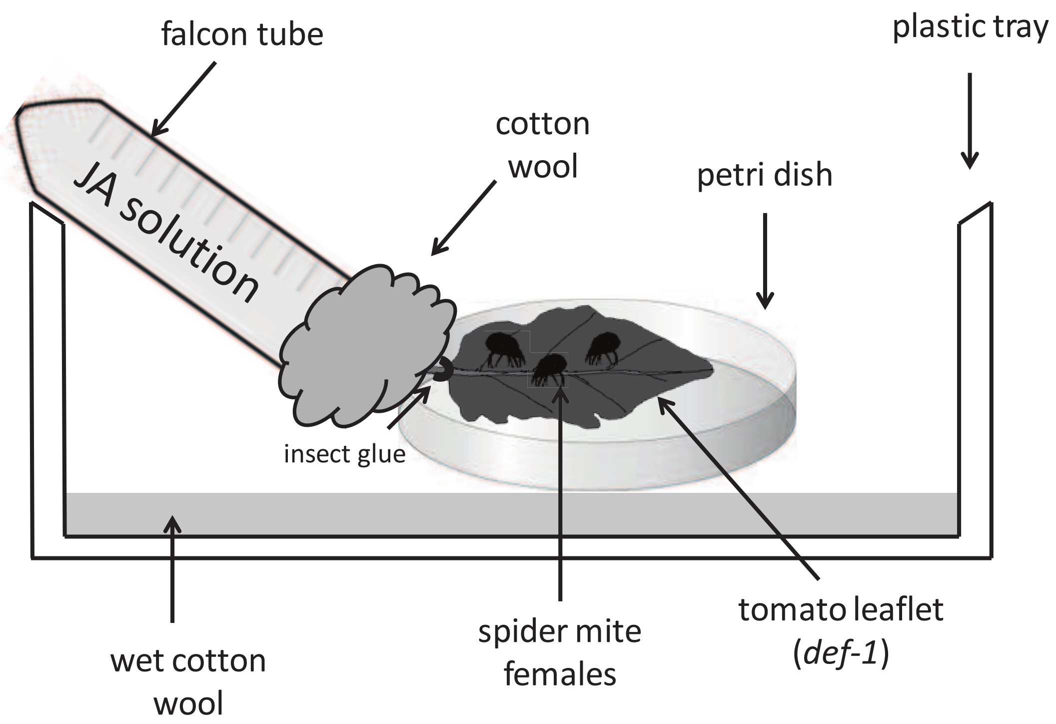

Figure 1 – Schematic drawing showing the set up used to evaluate the effect of the JA defences on the reproductive performance of the spider mites *T. evansi* and *T. urticae* and the predatory mite *P. longipes*. First, a conical centrifuge tube (15 mL) was filled with one of the tree solutions (see methods) and the petiole of one leaflet was inserted into the tube. The opening of the tube was plugged with a piece of cotton wool to ensure that the leaflet petiole would be in contact with the solution. Plastic trays (40 x 20 cm) were covered with wet cotton wool (to prevent the spider to move to other leaflets) and Petri dishes (diam.= 55 mm) were placed in these plastic trays. The leaflets, plugged in one of the sides of the conical centrifuge tubes, were gently placed in the Petri dishes, with the tubes at an angle of 15 degrees with the side of the petiole pointing down to ensure that the petiole of the tomato leaflet would always have access to the solution. 15 adult female spider mites of either *T. urticae* or *T. evansi* were transferred to each leaflet where they were allowed to feed and reproduce for 48h. Leaflets receiving the three treatments were kept in separate trays.

Phytohormone analysis

Around 200 mg of frozen leaf material was grounded in 1ml of ethyl acetate spiked with JA-D5 and SA-D6 as internal standards at a final concentration of 100 ng ml-1. Plant material was removed by centrifugation at 13000 rpm during 20 minutes. Supernatants were transferred to a new tube. To increase the recovery rate, the remaining plant material was cleaned with ethyl acetate without internal standards. The ethyl acetate fractions were pooled and evaporated in a concentrator (Labconco) at room temperature. 500 µl of methanol 70% was used to re-dissolve phytohormones and internal standards.

Samples were analyzed with Liquid Chromatography (Varian ProStar) using a column chromatography Pursuit 5 C18 50x2.0 mm (Varian) coupled to a double Mass Spectrometer in tandem (Varian 320 MS-MS, http://varianic.com). Serial dilutions of pure compounds of JA, JA-Ileu and SA were used to estimate hormone concentrations and retention times. JA-D5 and SA-D6 values were used to calculate the recovery rate and correct the hormone concentration. The hormone concentration was estimated through integration of peak areas, corrected for the fresh weight of leaflet tissue. Hence, the results are given in ng per gram of fresh weight (ng/g FW).

Effect of the jasmonate defences on predator performance

We offered the eggs of 15 adult spider mite females (*T. urticae* or *T. evansi*) produced on leaflets that received the treatments described above to the predatory mite *P. longipes*, to investigate the effect of JA-induced plant defences on its performance. The spider mites were removed from the leaflets with a paintbrush, leaving their eggs. One female predator (8 days old) was placed on the leaflet and allowed to feed on the spider mite eggs. After 48h, the predation rate of each female was assessed. Thereafter, they were removed and placed on new leaflets that received the same treatments and contained spider mite eggs, where they were allowed to feed for another 48h and then their fecundity was assessed.

Effect of the jasmonate defences on predator preference

Because spider mite feeding on wild type (wt) tomatoes (Castlemart) induces JA accumulation and it does not in *def-1*, we offered the predator a choice between spider mite eggs reared on these two plants. The aim of this experiment was to evaluate if due to a net effect, the induction of plant defence would affect the choice of the predator.

Since the wild type and *def-1* tomato plants were included as a treatment in this experiment, we could not have used one of these plants as a substrate for spider mites oviposition. Otherwise, instead of 48h of spider mite feeding, for one of the treatments mites would have been feeding for 72h. Therefore, we chose bean leaves as a “neutral” substrate for spider mite oviposition and subsequently offered the eggs on these bean leaves to the predatory mites.

Using bean leaves as a common substrate for all the mites we excluded the possibility that the predatory behaviour would be affected by the bean itself. Attempts to use an artificial substrate were done, but the mites did not lay enough eggs, possibly due to absorption of eggs by the starving female. Transferring the eggs from tomato plants to an artificial substrate is not reasonable, because most of the eggs are damaged by the paintbrush.

First, we allowed *T. evansi* and *T. urticae* to feed on wild type and *def-1* tomato plants for 48h. Subsequently, we made leaf discs (diam = 2.5 cm) from bean leaves (*Phaseolus vulgaris* L.) and divided each disc into two by putting a wet cotton wool barrier on the middle vein to prevent the mites to cross to the other side of the leaf. One of the disc halves was infested with 15 adult female spider mites that had previously been reared on wild type tomatoes and the other half was infested with 15 females previously reared on *def-1* plants.

All spider mites were of the same age (two days after final moulting) and were left on these arenas for 24h, after which the number of eggs was recorded. Subsequently, the wet cotton wool barrier and the mites were removed and 60 mite eggs were left on each leaf disc half. One predatory female (8 days old) was placed at the centre of the leaf disc and was allowed to prey upon the eggs for 24h. The number of eggs consumed on each side of the leaf disc was assessed.

Statistical analyses

Effect of the jasmonate defences on the reproductive performance of spider mites - Values numerically distant from the rest of the data set are often observed in a LCMS run which can be caused by technical issues. Therefore, we used Dixon's *Q* test to identify and exclude outliers in the phytohormone data. The accumulation of the phytohormones JA, JA-Ileu and SA was log-transformed. The effect of the treatment (water, JA-free and JA-rescued) and the spider mite species on the accumulation of all three phytohormones was analysed with a MANOVA. In this analysis, all the phytohormones were included as response variables and the treatments and spider mite species were included as explanatory variables.

Since a group of five leaflets were collected from the same tomato plant (*def-1*) and the experiments were carried out in two blocks in time, plant and block were included in the model as random factors. Thus, initially differences in the accumulation of each phytohormone among the treatments were analysed using linear mixed-effects models (nlme). Because model simplification showed that plant and block had no significant effects in the phytohormone accumulation, these two random factors were therefore, excluded from the analysis (Crawley, 2007). Hence, the effect of the treatment in the phytohormone accumulation was analysed using generalized linear models (GLM) with a normal error distribution. Treatment was included in the model as explanatory variable and phytohormone accumulation was log-transformed and included as response variable. Eight to ten replicates were done for each treatment.

To investigate which phytohormone explained most of the variation in spider mite reproductive performance, the oviposition rate of the spider mites was correlated with the phytohormones concentrations. To calculate the oviposition rate, which corresponds to the average number of eggs laid per female and per day, we divided the total number of eggs found on each leaflet by the number of females still present on these leaflets at the end of the experiment. The number of eggs laid per female was then divided by the number of hours that the females were allowed to feed on each leaflet and to calculate the number of eggs laid per day, the number of eggs laid per hour was multiplied by 24.

Initially, the oviposition rate of each spider mite was analysed using linear mixed-effects models (nlme) including the phytohormones JA, JA-Ileu and SA in the model as fixed effects and plant and block as random factors. Because model simplification showed plant and block had no significant effects in the phytohormone accumulation, these two random factors were excluded from the analysis (Crawley, 2007). Hence, the effect of the phytohormones in the reproductive performance of the spider mites was analysed using GLM with a normal error distribution, corrected for overdispersion using quasi-likelihood.

Subsequently, dose-response curves of the hormone that most explained the variation in the reproductive performance of the spider mites were fitted using Non-linear regression analyses (nls) with a normal error distribution. Differences in the reproductive performance of the spider mites among the treatments were checked using GLM with a normal error distribution corrected for overdispersion using quasi-likelihood.

Effect of the jasmonate defences on predator performance - Differences in the predation rate of *P. longipes* on spider mite eggs from leaflets among different treatments were initially analysed using linear mixed-effects models (nlme) including plant and block as random factors. 20 to 27 replicates were done for each treatment (water, JA-free and JA-rescue) in four blocks in time. Because plant and block had no significant effects in the predation rate of *P. longipes*, these two random factors were excluded from the analysis (Crawley, 2007). Hence, the predation rate was analysed using GLM with a quasi-Poisson error distribution and were included in the model as response variable and treatment as explanatory variable. The predation rate corresponds to the average number of eggs eaten per female and per day. Each replicate consisted of one predatory female feeding on spider mite eggs laid on one of the five leaflets collected from one plant submitted to the treatments detailed above.

Differences in the fecundity of *P. longipes* after feeding on *T. evansi* or *T. urticae* eggs produced on water, JA-free and JA-rescued treatments were analysed using generalized linear mixed-effects models (lmer) with Poisson error distribution including plant and block as random factors and treatments as fixed effects. Each replicate consisted of the fecundity of one predatory female after feeding on spider mites eggs produced on one of the five tomato leaflets collected from one plant submitted to the treatments detailed above. 16 to 26 replicates were done for each treatment (water, JA-free and JA-rescue) in four blocks in time.

Effect of the jasmonate defences on predator preference - The differences in the predation rate of *P. longipes* on *T. evansi* or *T. urticae* eggs were analysed separately for each spider mites species, using a replicated goodness-of-fit test (Sokal & Rohlf, 1995). Each replicate consisted of the predation rate of one predator on a bean leaf with two groups of mite eggs, i. e. eggs from mites reared on *def-1* and eggs from mites reared on wt plants. For predation on *T. evansi* eggs were carried out 25 replicates and for predation on *T. urticae* eggs 12 replicates, which were done in two blocks in time.

All these analyses were done for each spider mite species separately. Models were checked with residual analyses and eventually corrected for overdispersion as well as for the correctness of the assumed distribution. Contrasts among treatments were calculated by aggregating non-significant ($p>0.05$) treatment levels in an *a posteriori* stepwise procedure. All statistical analyses were done in R statistical software, version 2.15.1 (R Development Core Team, 2010).

Results

Effect of the jasmonate defences on the reproductive performance of spider mites

The JA treatment significantly affected the accumulation of all the phytohormones (MANOVA: Pillai-Bartlett statistic = 1.1; df= 2,6; $P< 0.001$, Figure 1). The effect on the accumulation of each phytohormone separately was also significant (ANOVA: JA: $F_{[2,47]}= 334.4$; $P< 0.001$; JA-Ileu: $F_{[2,47]}= 106.1$; $P< 0.001$; SA: $F_{[2,47]}= 9.9$; $P< 0.001$). In addition, the accumulation of the phytohormones JA (GLM: *T. evansi*: $F_{[1,23]}= 512.9$; $P< 0.001$; *T. urticae*: $F_{[1,27]}= 218.0$; $P< 0.001$) and JA-Ileu (GLM: *T. evansi*: $F_{[1,24]}= 135.7$; $P< 0.001$; *T. urticae*: $F_{[1,26]}= 66.0$; $P< 0.001$) occurred only in JA-rescued *def-1* leaflets. Thus, the accumulation of the phytohormones JA and

JA-Ileu only occurred when JA and Ileu were added simultaneously in the solution. Leaflets from JA-free and water treatments showed similar JA (GLM: $F_{[1,22]}$= 1.2; P= 0.294 for *T. evansi*; $F_{[1,26]}$=1.1; P=0.308 for *T. urticae*) and JA-Ileu accumulation (GLM: $F_{[1,23]}$= 0.2; P= 0.648 for *T. evansi*; $F_{[1,25]}$= 1.9; P= 0.176 for *T. urticae*).

The accumulation of the phytohormone SA follows the opposite trend as observed for JA and JA-Ileu. Leaflets with the addition of JA in the solution (JA-rescued) accumulated less SA than leaflets without the addition of JA (GLM: $F_{[1,24]}$= 5.9; P= 0.023 for *T. evansi*; $F_{[1,27]}$= 17.0; P< 0.001 for *T. urticae*). SA accumulation in leaflets from JA-free and Water treatments did not differ significantly (GLM: $F_{[1,23]}$= 0.3; P= 0.566 for *T. evansi*; $F_{[1,26]}$=0.2; P= 0.643 for *T. urticae*).

Interestingly, the accumulation of the phytohormones was also significantly affected by the species of spider mite feeding on the leaflets (MANOVA: Pillai = 0.2; df= 1,3; P= 0.01). Only JA accumulation was not affected by the spider mite feeding (ANOVA: JA: $F_{[1,47]}$= 2.2; P= 0.140; JA-Ileu: $F_{[1,47]}$= 8.1; P< 0.006; SA: $F_{[1,47]}$= 5.2; P< 0.027).

The reproductive performance of *T. evansi* and *T. urticae* was mostly correlated with JA-Ileu (Figure 2). The phytohormones JA and SA were not correlated with significant differences in the reproductive performance of *T. evansi* (GLM: JA: $F_{[1,20]}$= 0.2; P= 0.698; SA: $F_{[1,22]}$= 0.1; P= 0.804) or *T. urticae* (GLM: JA: $F_{[1,25]}$= 2.8; P= 0.111; SA: $F_{[1,24]}$= 1.8; P= 0.200). Both spider mite species laid significantly fewer eggs when the levels of JA-Ileu increased (GLM: $F_{[1,23]}$= 4.9; P= 0.036 for *T. evansi*; $F_{[1,26]}$= 15.8; P< 0.001 for *T. urticae*, Figure 2).

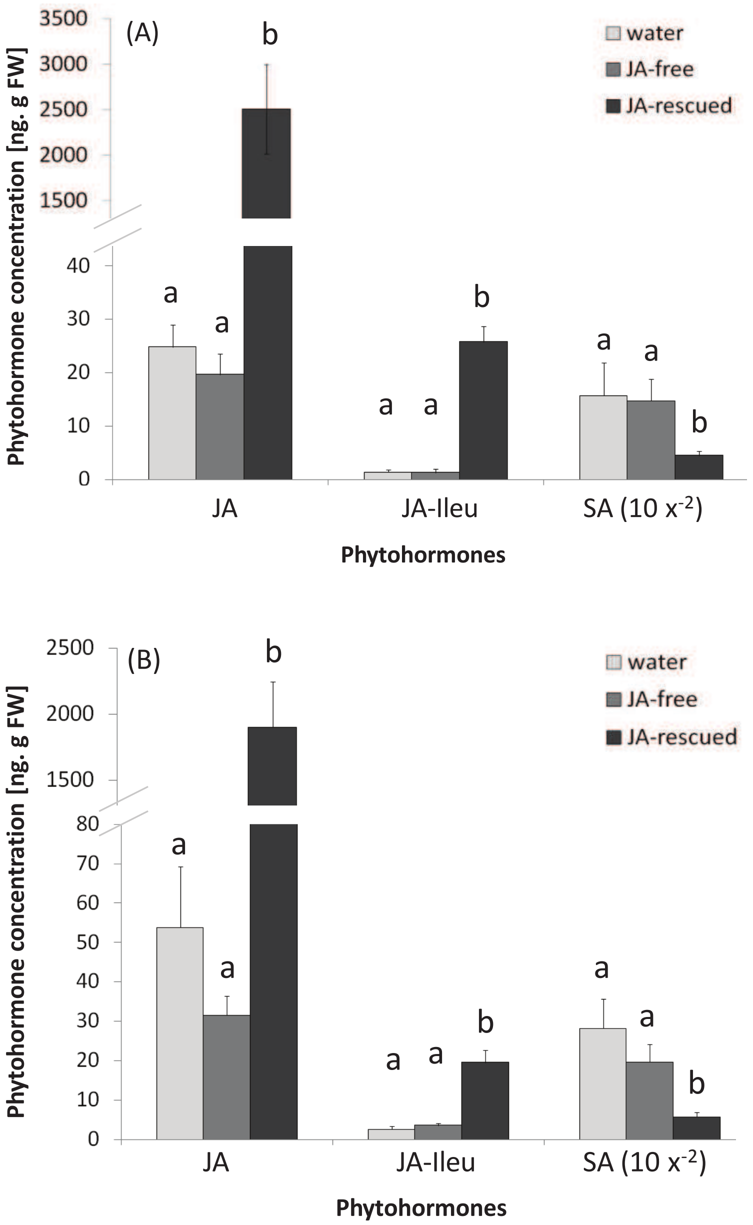

Figure 1 – The effect of the JA treatment on phytohormone accumulation (ng.g FW) in mutant tomato (*def-1*) leaflets infested with *T. evansi* **and** *T. urticae***.** Tomato leaflets were treated only with water (light gray bars), only with Ileu (dark gray bars) or with JA plus Ileu (black bars) and offered to 15 *T. evansi* **(A)** or *T. urticae* **(B)** females (2 days old) for 48h. Different letters within each phytohormone denote significant differences ($P < 0.05$) among treatments.

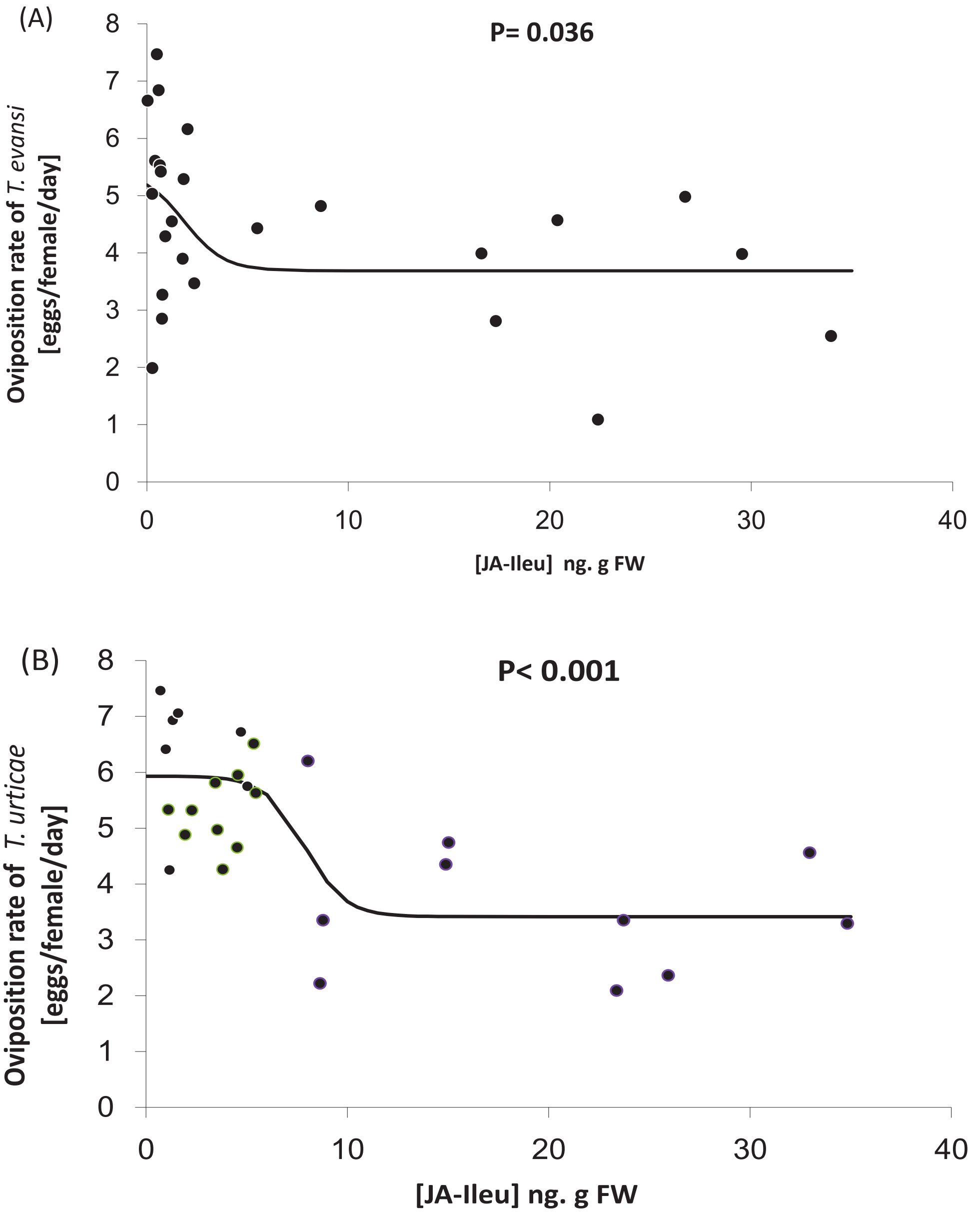

Figure 2 – The effect of the JA-Ileu accumulation in the reproductive performance of spider mites. 15 spider mites females (2 days old), either *T. evansi* **(A)** or *T. urticae* **(B)** were placed on mutant (*def-1*) tomato leaflets treated or not with JA. After 48h the spider mite oviposition and the phytohormone accumulation were assessed in those leaflets. The oviposition rate of *T. evansi* (P= 0.036) and *T. urticae* (P< 0.001) was negatively affected by the increase in the JA-Ileu accumulation.

Effect of the jasmonate defences on predator performance

The predation rate of *P. longipes* on spider mite eggs was influenced by the leaflet treatment (GLM: $F_{[2,72]}$= 6.0; P= 0.003 for eggs of *T. evansi* and $F_{[2,66]}$= 6.8; P< 0.001 for eggs of *T. urticae*). Predation was significantly lower on eggs oviposited by females that came from JA-rescued leaflets than on leaflets receiving the other treatments (Figure 3, GLM: $F_{[1,73]}$= 11.5; P= 0.001 for *T. evansi* and $F_{[1,67]}$= 13.5; P< 0.001 for *T. urticae*). The predation rate did not differ significantly between the JA-free and water treatments (GLM: $F_{[1,72]}$= 0.6; P= 0.439 for *T. evansi* and $F_{[1,66]}$= 0.2; P= 0.664 for *T. urticae*).

The fecundity of the predators after feeding on *T. evansi* eggs was not affected by the previous spider mite diet (Figure 4). It indicates that *P. longipes* oviposition was not significantly different after feeding on *T. evansi* eggs produced on JA-rescued, JA-free or water treatments (lmer with $Chi_{[2,4]}$= 0.04; P= 0.98). However, *P. longipes* fecundity after feeding on *T. urticae* eggs was affected by the previous *T. urticae* diet (lmer: $Chi_{[2,4]}$= 7.5; P= 0.02; Figure 4). Predatory females laid fewer eggs after feeding on eggs that came from JA-rescued leaflets than from JA-free or water leaflets (lmer: $Chi_{[1,3]}$= 6.9; P= 0.008). The numbers of eggs laid on JA-free and water leaflets did not differ significantly (lmer: $Chi_{[1,4]}$= 0.6; P= 0.454).

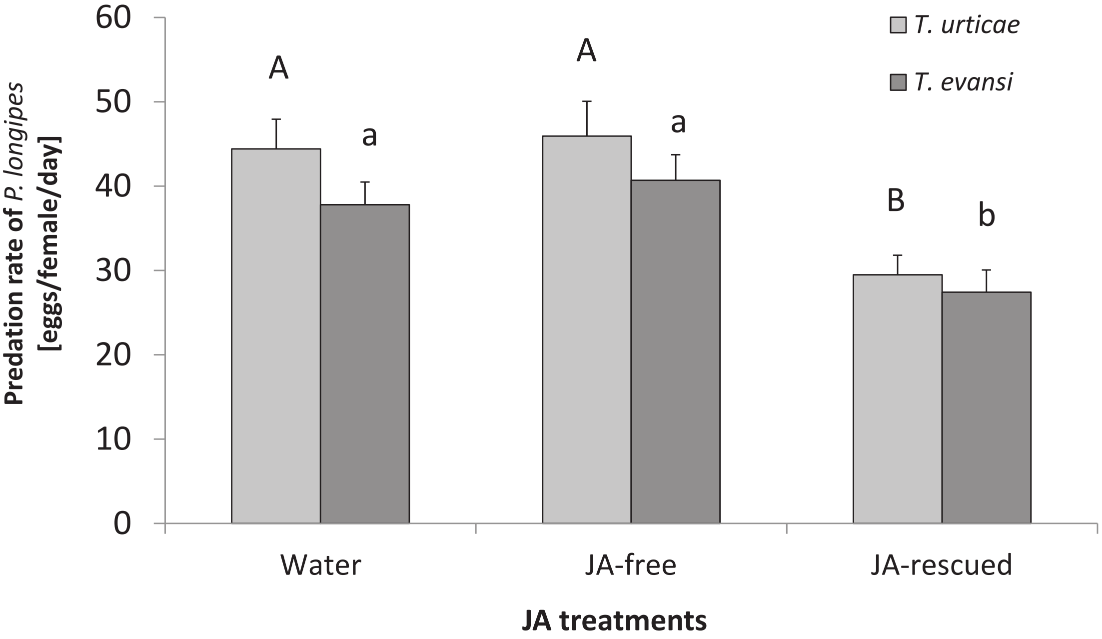

Figure 3 – *P. longipes* predation on spider mite eggs produced on leaflets under JA treatment. One predatory female (8 days old) was placed on the leaflet and allowed to feed on *T. evansi* (dark gray bars) or *T. urticae* (light gray bars) eggs produced on mutant (*def-1*) tomato leaflets treated only with water (water), only with Ileu (JA-free) or with JA plus Ileu (JA-rescued). After 48h, the predation rate of each female was assessed. Different capital letters represent significant differences (P< 0.05) in *P. longipes* predation on eggs produced by *T. urticae* on leaflets under the treatments and different small letters represent differences in *P. longipes* predation on eggs produced by *T. evansi*.

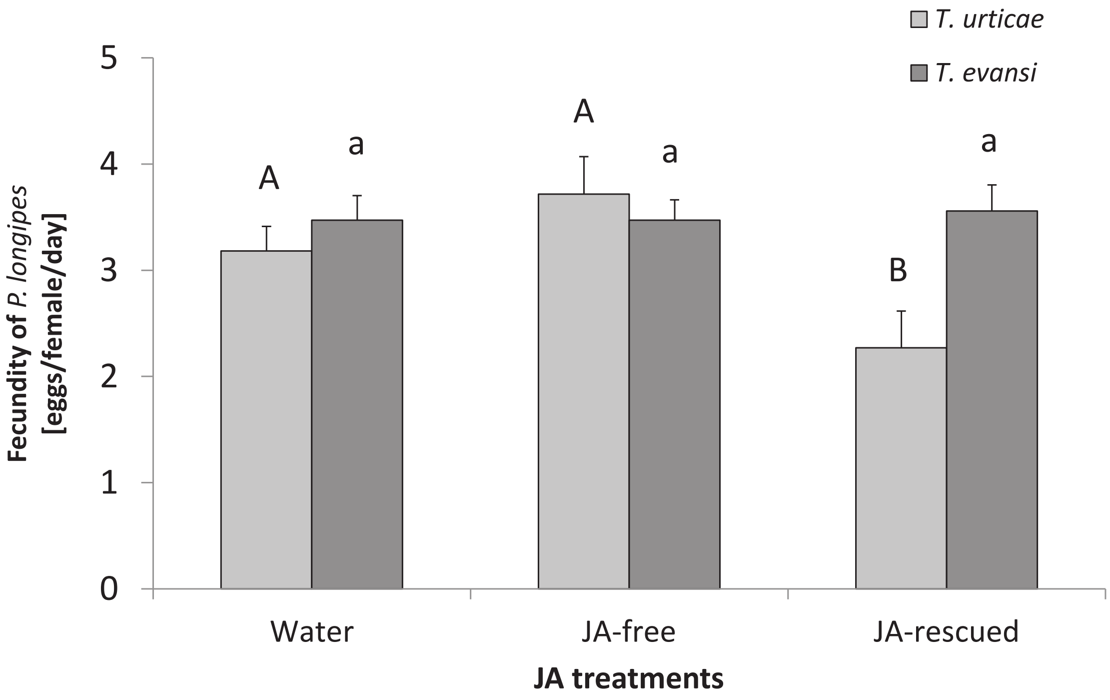

Figure 4 – *P. longipes* fecundity after feeding on spider mite eggs produced on leaflets under JA treatment. One predatory female (8 days old) was placed on the leaflet and allowed to feed on *T. evansi* (dark gray bars) or *T. urticae* (light gray bars) eggs produced on mutant (*def-1*) tomato leaflets treated only with water (water), only with Ileu (JA-free) or with JA plus Ileu (JA-rescued) for 48h. The number of eggs laid per predatory female after preying on these eggs was assessed after another 48h. Different capital letters represent significant differences ($P < 0.05$) in *P. longipes* fecundity after preying on eggs produced by *T. urticae* on leaflets under the treatments and different small letters represent differences in *P. longipes* fecundity after preying on eggs produced by *T. evansi*.

Effect of the jasmonate defences on predator preference

The predator preference for spider mite eggs was influenced by the spider mite feeding on JA defended or undefended plants. When we offered eggs from *T. evansi* reared on *def-1* plants and on wild type plants, we observed a significant heterogeneity in the choice of the predators by eggs from these two treatments (G_H= 209.0; *df*=22; P< 0.001) and the predators preyed similar amount of eggs from both treatments (G_p= 0.3; *df*=1; P= 0.59, Figure 5a).

T. evansi suppresses JA plant defences in wild type plants (Sarmento *et al.*, 2011a) and jasmonate-induced response is absent in *def-1* plants. Therefore, *T. evansi* eggs offered to the predators were produced on plants without JA induced defences. It suggests that *P. longipes* perceived that eggs from both treatments came from JA undefended plants and hence, preyed equally well on those eggs.

P. longipes preferred to prey on eggs from *T. urticae* reared on *def-1* plants over those from wild type plants (G_p= 36.7; *df*=1, P< 0.001, Figure 5b). However, as well as found for *T. evansi* eggs, there was a significant heterogeneity among the choice of the predators by eggs from these two treatments (G_H= 54.4; *df*=11; P< 0.001).

Although the choice of the predators was not homogeny among the replicates, our results suggest that the predators can discriminate among *T. urticae* eggs produced on JA defended and JA undefended plants. Since *T. urticae* highly induces the accumulation of JA defences in wild type plants, but does not in *def-1* plants (Li *et al.*, 2002; Ament *et al.*, 2004; Kant *et al.*, 2004), it indicates that the predators preferred to eat eggs from *T. urticae* reared on JA-undefended plants.

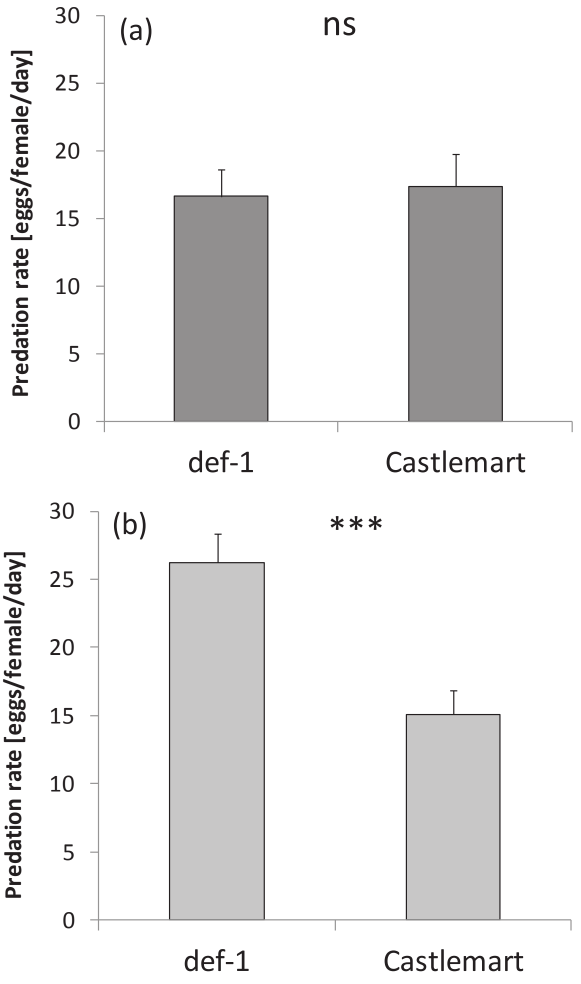

Figure 5 – Feeding of *P. longipes* on spider mite eggs reared on wild type tomato plants (Castlemart) and JA mutant tomato plants (*def-1*). Spider mite females were reared on wild type or *def-1* tomatoes for 24h and then removed from the plants and transferred to detached bean leaves to produce eggs. One female predatory mite was placed on each bean leaf to choose and prey on eggs from *def-1* and Castlemart treatments during 24h. **(a)** The predation rate of *P. longipes* on eggs from *T. evansi* and **(b)** on eggs from *T. urticae*. Tree asterisks (***) denotes P< 0.001 and (ns) denotes no significant difference (P> 0.05).

Discussion

Our results show that both spider mite species are negatively affected by JA-defences. In addition, when we offered eggs from spider mites reared on plants with high or low JA-defences to the predatory mite *P. longipes*, they ate more eggs from spider mites reared on plants with low JA-defences. These results suggest that eggs produced on plants with low JA-defences are more palatable to predators.

Jasmonates are involved in the induction of herbivore-induced defences in tomato; they regulate the accumulation of defensive metabolites and proteins, such as PIs (Farmer & Ryan, 1992) and PPO (Constabel *et al.*, 1995). JA exogenous application in leaf tissues (Cooper & Goggin, 2005; Bruinsma *et al.*, 2007; Bruinsma *et al.*, 2008) and defensive proteins regulated by JA (Green & Ryan, 1972; Ryan, 1990; Constabel *et al.*, 1995; Jongsma & Bolter, 1997; Lawrence & Koundal, 2002; Kant *et al.*, 2004) correlate negatively with herbivore performance.

The jasmonate mutant *def-1* is deficient in the biosynthesis of JA and therefore highly vulnerable to cell content feeders, while the mutant prosystemin, which over-expresses a 35S:prosystemin transgene that constitutively activates the JA pathway, is highly resistant (Li *et al.*, 2002). We demonstrate that we could restore the disability of the JA-biosynthesis mutant *def-1* to produce JA and JA-Ileu and to display the associated defences by placing leaflets into a watery solution containing JA and Ileu (Figure 1). Furthermore, our results also show that the feeding of *T. evansi* and *T. urticae* differently affected the accumulation of the phytohormones JA-Ileu and SA, indicating that not only the JA treatment, but also the spider mite feeding influenced the accumulation of phytohormones.

It is known that *T. evansi* is able to suppress tomato plant defences (Sarmento *et al.*, 2011a) and the defences otherwise induced by *T. urticae* (Alba *et al.*, in preparation). Therefore, the reason of why we chose to rescue the JA pathway in *def-1* mutant plants adding JA and Ileu, instead of using *T. urticae* to induce this pathway on wild type tomatoes was to assure that the JA-pathway would be induced and not be suppressed afterwards by *T. evansi*. Hence, the effect of the JA treatment would be quantitatively comparable among the treatments.

The different effects of the two spider mite species on phytohormone accumulation suggests that *T. urticae* and *T. evansi* feeding might interfere with the accumulation of the phytohormones beyond the levels rescued in *def-1* leaflets by the JA treatment. Nevertheless, the JA defences were only highly induced in JA-rescued leaflets (Figure 1) and we were able to evaluate the effect of the JA pathway on the reproductive performance of spider mites and of their predatory mite.

The phytohormone JA-Ileu is the major bioactive form of the hormone JA (Koo & Howe, 2012) and the expression of genes encoding for JA-related defensive proteins depends on the action of JA-Ileu. Alba *et al.*, (in preparation) has shown a positive correlation with leaflet contents of JA-Ileu and expression levels of genes encoding defensive proteins. Here we found that JA-Ileu was the hormone that most correlated with a negative effect in reproductive performance of spider mites.

Suppressor and inducer mites were negatively affected only by low levels of JA-Ileu accumulation (Figure 2). It indicates that there is no effect of JA-Ileu accumulation in spider mites reproductive performance when the levels highly increased, which means that for effective defence it is not necessary to increase JA-Ileu accumulation much over what is constitutively present in the plant. Similarly, exogenous application of JA in tomato plants has been found to restore its resistance against other herbivores, such as the caterpillars *Spodoptera exigua* (Thaler *et al.*, 2002) and *Mamestra brassicae* (van Dam & Oomen, 2008) and the aphid *Macrosiphum euphorbiae* (Cooper & Goggin, 2005). In addition, we found that the SA-levels of *def-1* leaflets decrease upon treatment with JA, probably due to the antagonistic effect of the second on the first (Mur *et al.*, 2006). To our knowledge there is no indication that SA defences as such can promote herbivore reproductive performance (i.e. independent from JA defences) and hence we believe that the significant differences of SA accumulation among the treatments (Figure 1) cannot be attributed to a direct causal relationship between SA and performance.

In addition to direct defences that reduce herbivore performance, the JA-signalling pathway is also involved in indirect defences. JA is involved in induced indirect defences in tomato as a precursor of plant-produced volatiles that attract or arrest natural enemies of herbivores for example via plant volatiles or alternative food (Thaler, 1999; Thaler *et al.*, 2002; van Poecke & Dicke, 2002; Bruinsma *et al.*, 2008) and JA deficient plants have reduced indirect defences against herbivores, which can be rescued after JA treatment (Dicke *et al.*, 1999; Gols *et al.*, 1999; van Poecke & Dicke, 2002; Bruinsma *et al.*, 2009).

Since JA orchestrates the interaction among plants, herbivores and carnivores, it can influence natural enemies in different ways. It has been shown that induced plant resistance can have a negative effect on natural enemies through altered host quality (Barbercheck *et al.*, 1995; Traugott & Stamp, 1997; Thaler, 1999; Gassmann *et al.*, 2010). Similarly, here we infer that restoration of JA plant defences correlated negatively with predation rate and preference of *P. longipes* (Figure 3 and 5, respectively). Additionally, we also demonstrate a negative effect of JA-defences on predator's fecundity because *P. longipes* lays fewer eggs after eating *T. urticae* eggs that came from JA-rescued leaflets than from non-rescued leaflets (Figure 4).

Considering that compounds that function as direct plant defences against herbivores can be sequestered by the herbivores and used against natural enemies to reduce their performance (Reitz & Trumble, 1997; Havill & Raffa, 2000; Soler *et al.*, 2007; Chaplin-Kramer *et al.*, 2008), this result suggests that *T. urticae* sequestered JA or other defensive plant compounds from JA defended leaflets and that such compounds were transferred to its eggs, thereby affecting the fecundity of *P. longipes*.

JA has been found in eggs of 15 families of insects belonging to nine different orders (Tooker & De Moraes, 2007). However, the function of JA in these eggs is as yet unknown, but it could have a signalling or defensive role (Tooker & De Moraes, 2007). Therefore, if it is true that defensive compounds can be found in spider mites eggs and that it could have defensive roles, it is possible that it affects their natural enemies.

The decreased predation of spider mite eggs can perhaps be attributed to such defensive compounds in the eggs, originating from the host plant (Figure 3). Therefore, the poor nutritional state of the eggs produced on plants with JA would explain why feeding on these eggs triggered a direct effect on *P. longipes* fecundity (Figure 4). Further research is underway to investigate the presence of defensive compounds in spider mite eggs and their potential role in defence against predatory mites.

We also show here that predator's fecundity is differently affected after feeding on *T. urticae* or *T. evansi* eggs (Figure 4). *P. longipes* laid similar amount of eggs after eating *T. evansi* eggs that came from JA-rescued or non-rescued leaflets. Hence, although *P. longipes* ate fewer *T. evansi* eggs from JA-rescued leaflets (Figure 3), its fecundity was not lower on these leaflets (Figure 4). In this case, the predator's fecundity was not negatively affected by JA defences and it even suggests that eggs produced on plants with JA were most efficiently used by the predatory mites.

Plant defensive compounds sequestered by herbivores can be used against natural enemies and generalists may suffer more with the presence of these compounds than specialist predators (Barbosa *et al.*, 1986). Because *P. longipes* is a predatory mite that has been found to be associated with *T. evansi* (Furtado *et al.*, 2006), but not with *T. urticae* in South Brazil, it suggests that the net effects of the JA would have less severe effects after feeding on *T. evansi* eggs than after feeding on *T. urticae* eggs.

In summary, we have shown here that JA-induced tomato plants not only decreased spider mite performance, but also decreased the rate by which their eggs were eaten by predatory mites. This suggests that the costs of inducing defences, i.e. a lower reproductive performance, may be (partly) compensated by the fact that natural enemies are affected by these defences as well and, therefore eat less of these eggs.

We suggest that the induction of plant defences would be a positive outcome for the inducer mite *T. urticae*, which would be less susceptible to predation and predator's fecundity would be lower when feeding on these eggs although it would lay fewer eggs on induced plants. In contrast, suppression of plant defences by *T. evansi* would improve mite performance, but would make them more vulnerable to predators, because predators would eat more eggs and have higher fecundity. However, more research is needed to evaluate the consequences of plant defence suppression by *T. evansi* and how suppression affects the population dynamics of predator and prey involved in this interaction.

Acknowledgements

We thank CAPES, CNPQ and FAPEMIG for financial support. We also thank Gregg Howe (122 Plant Biology Lab, Michigan State University, USA) who kindly provided the *def-1* mutant tomato plants.

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General conclusions

In this thesis I have studied the molecular machinery of herbivore defence suppression and induction as well as its ecological consequences, aiming to bridge ecology and molecular biology. These two fields of research traditionally work separately in the science. To bring them together can provide novel insights into how to use and breed tomato plants and how to manipulate the natural enemies to favour pest control in agriculture.

If it was already known that *T. evansi* is able to suppress plant defences on tomato, in chapter 2, I showed that *T. evansi* also manipulates plant defences on beans, which can be an alternative plant for reproduction, suggesting that suppression is a wide spread mechanism used by this herbivore. Therefore, *T. evansi* remarkably employs different strategies from *T. urticae* and most herbivores to cope with plant defence. Its ability to manipulate plant defences makes this herbivore and its attacked plants a suitable model system to study, for example, the role of variation among individuals. This knowledge can contribute to understand the frequent adaptations of pests to pesticides and how we can utilize natural plant defences against pests in agriculture. In addition, to clarify how herbivores deal with plant defences can be relevant for the pest management using resistant plants. For instance, since some pests can resist or suppress plant defences the introduction of plants with resistant traits such as continuous synthesis of defensive compounds might not be efficient in controlling them.

In chapter 1 and 3, I have studied the net effects of suppression by *T. evansi* and induction by *T. urticae*. My data demonstrates the importance of studding the net effects of these two strategies (suppression and induction) before the introduction of natural enemies as a tool in the biological control of *T. evansi* and *T. urticae*. If we suppose that natural enemies can be indirectly affected by plant defences, suppression might benefit the plant and the predator and it may consist in a “plus” for pest control. However, if the suppression only occurs at the feeding site of the herbivore and it prevents the natural enemies to reach their colonies by producing a dense web, the herbivore will fast overexploit the plant and it may consist in a “minus” for pest control. Hence, to better understand the mechanisms, consequences and implications of suppression and induction of plant defences by herbivores; plant, herbivores and natural enemies cannot be independently studied anymore. Likewise, ecologists and molecular biologists should keep on working together to provide a full overview of the system and to find new alternatives to control pests in agriculture.